Differential expression of phosphorylated mitogen-activated protein kinase (pMAPK) in the lateral amygdala of mice selectively bred for high and low fear

by

Jennifer L. Coyner, LTC, US Army Nurse Corps

Dissertation submitted to the Faculty of the
Graduate Program in Neuroscience
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2013

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

DISSERTATION APPROVAL FOR THE DOCTORAL DISSERTATION IN THE NEUROSCIENCE GRADUATE PROGRAM

Title of Dissertation: "Differential expression of phosphorylated mitogenactivated protein kinase (pMAPK) in the lateral amygdala of mice selectively bred for High and Low Pavlovian conditioned fear"

Name of Candidate: LTC Jennifer Coyner
Doctor of Philosophy Degree
July 2, 2013

DISSERTATION AND ABSTRACT APPROVED:

DATE:

Gregory P Mueller 7/17/2013
Dr. Gregory Mueller
DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS
Committee Chairperson

7/17/2013
Dr. Luke Johnson
DEPARTMENT OF NEUROSCIENCE
Dissertation Advisor

Sharon L Juliano 7/17/13
Dr. Sharon Juliano
DEPARTMENT OF ANATOMY, PHYSIOLOGY, AND GENETICS
Committee Member

Brian Cox 8/12/13
Dr. Brian Cox
DEPARTMENT OF PHARMACOLOGY
Committee Member

Roopa Biswas 7/17/13
Dr. Roopa Biswas
DEPARTMENT OF ~~PATHOLOGY~~ ANATOMY, PHYSIOLOGY & GENETICS
Committee Member

7/17/13
Dr. Suzanne Bausch
DEPARTMENT OF PHARMACOLOGY
Committee Member

COPYRIGHT STATEMENT

The author hereby certifies that the use of any copyrighted material in the thesis manuscript entitled: "Differential expression of phosphorylated mitogen-activated protein kinase (pMAPK) in the lateral amygdala of mice selectively bred for high and low fear" is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

Jennifer L. Coyner, LTC, US Army
Program in Neuroscience
Uniformed Services University
06/21/3013

ACKNOWLEDGMENTS

The successful completion of a doctoral dissertation is largely an individual journey in which our days and much of our time and focus are spent seeking to add to an existing body of knowledge. We choose a question that has not been answered and it is one in which (hopefully) we are passionately interested. The hours turn into years, and the learning is more diverse than you had any idea it would be. In some ways, the learning and growth that occurs is hard to describe in words. We think differently. Our perception of how things are and can be is altered. To a certain degree, we become far more critical and skeptical. That is both good and bad. I am so pleased to have completed this work and I did not get to the finish line on my own. So it is here that I wish to formally acknowledge those who helped me in various ways either directly or indirectly.

I would first like to thank the Army Nurse Corps (ANC) for allowing me the opportunity to complete this degree without the additional challenge of "working" full or part-time. I have always been extremely grateful for the educational opportunities the ANC provides and actually encourages its officers to seek. While I am certain that we have a "win-win" situation, I will always feel as though I got the better end of the deal. I simply hope that my service does justice to the many ways in which the ANC has served me.

To COL Bruce Schoneboom, I am indebted to you for all of your formal and informal mentoring and support. You push, you encourage, and you absolutely set the example. You were instrumental in my decision to seek this "highest of degrees" and

your guidance during my first year of the program was tremendous. Thank you for your continued mentoring and inspiration.

To COL Paul Lewis, I will forever be grateful for your leadership, mentoring, support, wisdom, and for the ways in which your kindness and willingness to listen aided me personally, professionally, and academically. Our formal and informal conversations enriched me as an officer, a leader, and as a researcher. You expressed a genuine interest in my field of research and often served to "recharge" my enthusiasm for the important topic of fear memory. I will miss our discussions on so many diverse topics. When I move on to this next chapter in my career, it will undoubtedly be you that comes to mind when a student comes to me with a question or an "issue". I hope that I will be able to impart the same level of mentorship you have consistently given to me. My sincerest thanks, sir....

I wish to thank the Tri Service Nursing Research Program (TSNRP) for funding my research through a Graduate Research Award. I am a proud military nurse and it's an honor to be part of such a great organization. I look forward to future collaborations with TSNRP.

To Dr. Luke Johnson, I am grateful for the trust you placed in me to conduct research in your lab. I was drawn to your lab from the beginning and we have enjoyed a solid partnership. I appreciate your work and your passion for this field of research. Your calm, level-headedness combined with impressive knowledge has been good for me. I feel that I got the best of you when our meetings would roll into an informal "brainstorming session" and I had the opportunity to discuss all of the possibilities...that's really the good stuff in academics. You have been supportive of the

unique demands placed on a military graduate student and I appreciate that deeply. I wish you all the best as you move on in your career.

To Dr. Hadley Bergstrom and Dr. Jen McGuire, thank-you for your mentoring and support. I needed you. The ways in which you helped and inspired me can't really be put into words. You always took the time to answer a question or take a look at my data or troubleshoot a problem. It's fun to work around individuals who are passionate about what they do and the two of you (in somewhat different ways) fit that description.

Anna Sharova, you are part of my success and I am grateful for your assistance. You will do great at OSU!

To my dissertation committee: Dr.'s Greg Mueller, Suzanne Bausch, Roopa Biswas, Brian Cox, and Sharon Juliano, I sincerely appreciate the time you devoted to making sure I met the mark. Although I feel that much of the learning will actually begin once I'm done with this program (as is the case with much of school), I will always be grateful for the mentoring and advising I received from each of you. Sometimes a three-minute conversation had more impact than a week's worth of work.

To Dr. David Jacobowitz, I am honored to have had the opportunity to work with you and learn from you. You always showed me sincere kindness and respect and trust me, the feeling is mutual. I am taking important lessons with me thanks to your tutelage.

To all of the veterinarians, veterinary staff, animal husbandry division (AHD), and the IACUC, thank-you for taking care of our partners in research and serving as their advocate.

To my friends and colleagues at USU, your individual friendships mean the world to me and I will reflect on our good conversations for many years to come. To my "battle

buddy", you are a gem and I'm happy that we will be teaching together at our next assignment.

Finally, I want to acknowledge my family, as I always do…because whether they are directly or indirectly involved in what I am doing, they serve as my foundation and that is no small thing. They have very little concept of what I've been doing for the past 4 years, but they know I've worked awfully hard and have enjoyed very little "down time" throughout this academic pursuit. I believe I am finally done with school now. It's time to enjoy my family, friends, and my life…and it's time to get "back to work"!

DEDICATION

This work is dedicated to all persons living with fear-related illness. In adding to the body of knowledge related to neurobiological mechanisms underlying fear pathology, my hope is that one day soon we will be able to more effectively treat the underlying causes of fear pathology and assist in the return of healthy fear processing and responding. To the military service members living with the invisible wound of PTSD, you are my inspiration.

ABSTRACT

Differential expression of phosphorylated mitogen-activated protein kinase (pMAPK) in the lateral amygdala of mice selectively bred for high and low fear

Jennifer L. Coyner, MSN, 2002

Thesis directed by: Luke Johnson, Assistant Professor, Department of Psychology and The Center for the Study of Traumatic Stress, Uniformed Services University, Bethesda, Maryland

Post traumatic stress disorder (PTSD) is a serious medical condition affecting both military and civilian populations. While its etiology remains poorly understood PTSD is characterized by high and prolonged levels of fear response. The neuronal processing of fear occurs in the amygdala, a complex structure located in the temporal lobe of the brain. A known requirement for the long-term storage or consolidation of fear memory is the phosphorylation of mitogen activated protein kinase (p44/42 (ERK1/2) pMAPK) in the lateral amygdala (LA), a subnucleus of the amygdala. Increased expression of pMAPK in the LA is a reliable marker of the neuroplasticity underlying fear learning. One important biological unknown however, is whether individuals expressing high or low conditioned fear memory consolidate the memory differently, and if that difference underlies differences in fear response. A strategy for investigating this question is to examine the regional expression of pMAPK in the amygdala in animals that

exhibit high and low fear. Using a mouse model selectively bred to exhibit high and low fear, we used Pavlovian fear conditioning to examine pMAPK expression in the LA in these divergent lines of mice. We hypothesized that high fear mice would have greater pMAPK expression in the LA following fear conditioning as compared to low fear mice. Further, we hypothesized that pharmacologic inhibition of pMAPK in high fear mice would reduce fear memory strength to that of low fear mice. To examine these hypotheses, we quantified pMAPK-expressing neurons in the LA at baseline and at one-hour following fear conditioning. Results indicate that after fear conditioning, high fear mice have more pMAPK-expressing neurons in the dorso-lateral amygdala (LAd) a discrete subregion of the LA. We then used a selective inhibitor of the phosphorylation of MAPK prior to fear conditioning and examined its effects on fear memory strength and the quantity of pMAPK-expressing neurons in the LAd. The results indicate that inhibition of pMAPK reduces contextual and cued fear memory in high fear mice, and reduces contextual but not cued fear memory in low fear mice. Additionally, we found a dramatic decrease in pMAPK expressing neurons in the LAd of high fear mice in which MAPK phosphorylation was pharmacologically inhibited. This suggests that the reduced fear memory is due in part to decreased pMAPK in the LAd. These findings suggest that increased plasticity in the LAd is a component of higher conditioned fear responses and begins to explain, at the cellular level, how different fear responders may encode fear memories differently. Ultimately, this understanding may help to identify novel ways for both identifying and treating individuals who have developed fear-related disorders such as PTSD.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIL- advanced intercross line

AMPA-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA- analysis of variance

ASD- acute stress disorder

Ba/BA- basal nucleus of the amygdala

BEH- Behavior/Behavioral

BLA- basolateral nucleus of the amygdala

CBT- cognitive behavioral therapy

Ce/CeA- central nucleus of the amygdala

CR- conditioned response

CREB- cAMP response element-binding protein

CeM- medial Ce

CeC- central Ce

CS- conditioned stimulus

DMSO- dimethyl sulfoxide

ERK- extracellular signal-regulated kinase

IHC- immunohistochemistry

IACUC- institutional animal care and use committee

LA- lateral amygdala

LAd- dorso-lateral amygdala

LAvl- ventro-lateral amygdala

LAvm- ventro-medial lateral amygdala

LAM- laboratory animal medicine

LTP- long-term potentiation

MAPK- mitogen activated protein kinase

MRI- magnetic resonance imaging

NMDAR- N-methyl-D-aspartate receptor

PKA- protein kinase A

pMAPK- phosphorylated mitogen activated protein kinase

PTSD- Post-traumatic stress disorder

US- unconditioned stimulus

LIST OF FIGURES

CHAPTER 1: Introduction

Specific Aims of Research

1. Compare the quantity of pMAPK expressing neurons and patterns of expression of pMAPK expressing neurons (topography) in the lateral amygdala in naïve, tone control, and fear conditioned high and low fear phenotype mice.

2. Pharmacologically inhibit pMAPK signaling in High fear phenotype mice prior to fear conditioning and evaluate long-term fear memory

Overview

The study of the cellular and molecular mechanisms that underlie the formation of long-term fear memory is an important field in psychiatry and neuroscience. The formation of a memory to a threatening stimulus – a fear memory - is an important biological behavior necessary for survival, however pathology of these memories contribute to serious neurobiological illnesses such as post-traumatic stress disorder (PTSD) (55; 66). Documentation of one of the earliest experiments in the field of fear memory research was in 1920 when Watson and Rayner (133) taught an eleven month old infant ("Little Albert") to associate a soft white animal with a loud, aversive sound. Thereafter, presentation of the animal or any object resembling it elicited the same response in the child that resulted from the aversive noise (crying, withdrawal) (133). This highly cited experiment demonstrated that humans learn to apply new meaning to previously neutral stimuli if they are temporally linked with something aversive. This form of learning occurs every day and the associations we make are a major part of how we determine what is safe or unsafe in our world. Decades of research since this early

experiment have answered key questions related to mammalian fear circuitry, necessary signaling cascades, the impact of pharmacologic interventions, and how fear memories are extinguished (31; 55; 59; 74; 87; 111; 116; 119). Due to the highly shared fear circuitry among human and non-human animal species, research involving a diverse array of species has complementary benefits to the field and to our overall understanding of how our fear memories are formed (73; 114). Importantly, non-human animal models such as rodents allow us to attempt to answer questions we are unable to ask with human subjects and therefore, these animals serve as our partners in advancing science. There is great need for improved understanding of the mechanisms that take place during the acquisition and consolidation of a fear memory in high fear individuals. The work herein is the culmination of experiments aimed at unraveling mechanisms underlying divergent fear memory.

FEAR

Fear memories are an important component of PTSD (6; 47; 132). In humans, fear is an emotional response to a real or perceived threat (45). In non-human animals that are unable to verbalize what they are experiencing, the 'feeling' of fear is assumed to exist when defensive behaviors are witnessed (33; 73). All vertebrate and some invertebrate animals show evidence of fear when danger is sensed and this can be witnessed both in and out of the laboratory (73). Fear is evolutionarily-conserved, adaptive, and therefore it serves to promote survival (109). However, if exaggerated or inappropriate, fear can lead to pathology and disrupt the ability to function in society (11). Fear and anxiety-related illnesses place an enormous burden on individuals, families, communities, and systems of healthcare, costing our nation over $42 billion

each year (127). One objective of fear memory research therefore should be determining what mechanisms lead to exaggerated fear responses. In order to unravel the mechanisms of high fear memory, it is important to understand fear-related circuitry in the brain and examine the cellular processes involved in a fear response.

Fear memory

The ability to remember our most fearful experiences serves an important role in survival (45; 72; 109). Throughout history, living organisms exhibit both learned and unlearned (innate) defensive responses to threat which range from simple reflex withdrawal, avoidance behavior, fight or flight responses, to complex physiologic and motor responses as seen in higher mammals including humans (15; 17; 62; 73). All serve to aid the organism in avoiding harm and staying alive. Fear is an evolutionarily-conserved response that is adaptive and heritable (60). While crucial for survival, under certain circumstances, fear memories can be detrimental when they become generalized or lack precision (73). Understanding how and where the brain processes fear is crucial for understanding the cellular and molecular mechanisms that underlie the formation of a fear memory. This understanding will aid in the search for the identification of mechanisms that underlie pathological fear memory.

Fear Circuitry

Over the past several decades, our understanding of fear circuitry in the brain has markedly improved (2; 93). Key brain regions involved in the formation of a fear memory include the thalamus, hippocampus, and the amygdala (74). The fear circuit within the central nervous system is a complex network of connections between multiple structures, involving diverse signaling cascades (117). The thalamus serves as a critical

link between external stimuli and the fearful experiences an organism encounters throughout its life (61). Dense neuronal connections link the thalamus to both 'old brain' structures as well as the cerebral cortex allowing both automatic instinctual responses as well as informed deliberate responses among animals (61). The hippocampus plays a prominent role in contextual memory including the expression of contextual fear memory (81; 115). Conversely, extinction of a fear memory relies heavily on the connections between the amygdala and the pre-frontal cortex (86; 87). When humans hear, see, smell, or feel stimuli associated with a fear memory, that stimulus travels through the sensory-specific pathway to the thalamus and converges directly into the lateral amygdala (23; 75). That process occurs within approximately 12 milliseconds (75). This circuitry provokes the immediate response associated with a fearful situation (e.g. freezing) and is therefore said to occur in the absence of awareness, while a slower circuit occurs simultaneously and involves sensory and higher cortex (75). Fear responses in mammals are widely accepted to involve the amygdala (16; 36; 45; 70; 121). Animals with lesions to the amygdala show dramatically reduced fear (17). Likewise, animal models in which signaling through the amygdala is interrupted prior to fear conditioning show no evidence of fear learning (119). While much of the neural circuit that governs fear circuitry has been elucidated, (51; 74) important questions remain. Identifying processes involved in fear learning in high and low fear phenotype animals has the potential to improve the ways in which we examine and approach the treatment of human fear pathology.

THE AMYGDALA

The amygdala is known to play a critical role in the consolidation and expression of conditioned fear (31). The amygdala is a small structure in the temporal lobe of the brain that is composed of multiple subnuclei. Among these are the lateral, basal and central nuclei, which are understood to be involved in fear response and fear learning (2)). Cellular changes take place within the amygdala following associative learning (71; 80). Sensory stimuli associated with a fearful event rapidly converge in the lateral amygdala (LA) and initiate a signaling cascade leading to memory consolidation (54; 70; 113). A critical event in this cascade is the phosphorylation (activation) of mitogen activated protein kinase (pMAPK) (7; 119). Inhibitors of phosphorylation of MAPK in the amygdala of rodent models result in a deficit of fear memory consolidation (119; 123). Human and non-human animal neuroimaging studies indicate specific amygdala activity on provocation of fear exposure and learning (89). Further, the degree of amygdala activation correlates with the intensity of the conditioned fear response (20; 28). Additionally, lesions to the amygdala in both human and non-human animals consistently result in reduced fear and an inability to process fear emotion in self and others (2; 68). The last few decades of neurobehavioral research have greatly expanded our knowledge of fear circuits and mechanisms involved in the acquisition, consolidation and extinction of fear memory (93). However, many questions remain and treatments of fear-related pathologies such as PTSD are highly variable in their effectiveness (11). The cellular processes that take place in the amygdala are crucial to understanding the complex physiology of fear memory storage (135). Prolonged fear memory that leads to PTSD can potentially be managed early, thereby reducing the incidence and severity of this illness.

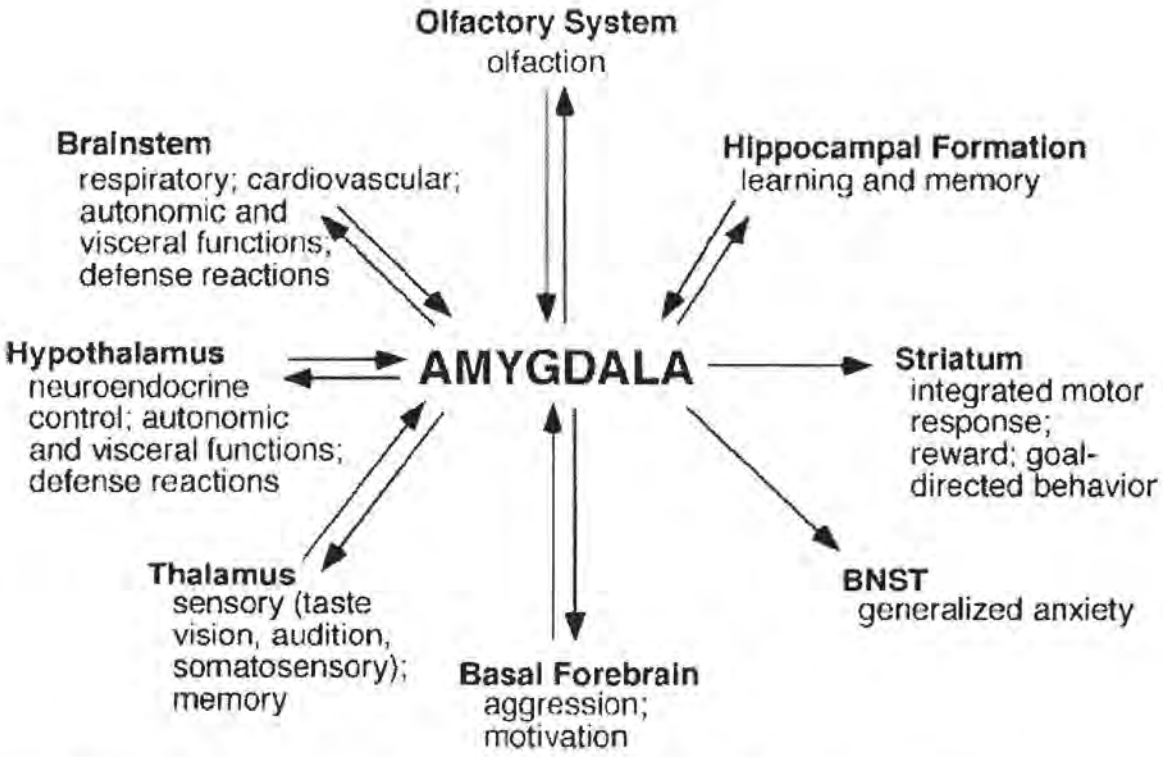


Figure 1.1. Schematic of the connectivity of the amygdala with diverse brain regions involved in the processing of fear memory. (136) (Whalen and Phelps, 2009)

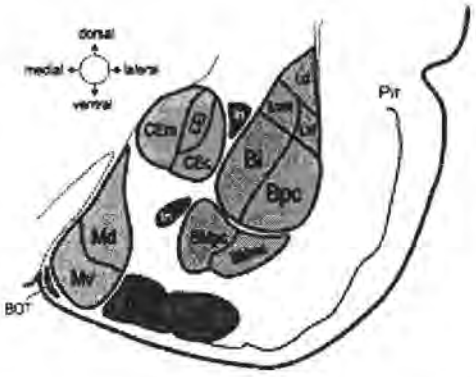


Figure 1.2. Coronal section of the mouse temporal lobe region demonstrating anatomical location of the amygdala. The amygdala is composed of approximately 12

subnuclei that have unique functions in fear processing as well as fear memory acquisition and storage. Ld= dorsolateral amygdala, Lvm=ventromedial lateral amygdala, Lvl=ventrolateral amygdala, Bi=intermediate division of basal nucleus, Bpc=parvicellular division of basal nucleus, Bmc=magnocellular division of basal nucleus, Bi, Bpc +Ld, Lvm, Lvl=BLA(basolateral amygdala), CEi= intermediate division of central nucleus, CEc=capsular division of central nucleus, CEm=medial central nucleus, COa=anterior cortical division, Cop=posterior cortical division, Md=dorsal medial division, Mv=ventral medial division (136) (Whalen and Phelps, 2009).

PTSD

PTSD is an anxiety disorder in which symptoms persist for more than one month and fall into one or more of three major categories: re-experiencing, avoidance, and hyper-arousal (6). Symptoms of PTSD are a result of an individual experiencing or witnessing events that pose a threat to their lives or could result in serious injury. Moreover, the individual experiences intense fear, helplessness, or horror (11). In the military population, post-traumatic stress disorder (PTSD) is a psychiatric injury that affects up to 18% of service members who have deployed or are deploying in support of our current operations (78). Combat exposure during deployment is associated with a markedly increased risk of developing PTSD (48; 102). The most reliable predictors of developing PTSD and other mental health illnesses in the military population are the frequency and intensity of combat experiences (48). Natural disasters, acts of terrorism, and violent physical abuse are common causes of PTSD in the civilian population (11). The effectiveness of treatment is highly variable, and typically occurs after PTSD has been diagnosed (11). Cognitive behavioral therapy (CBT) is the treatment of choice for PTSD, however, approximately half of patients do not respond to this modality (25). Functional MRI studies suggest that increased activity in the amygdala during fear

processing is associated with poor response to CBT (25). Fear is a major component of the development of PTSD (45). Thus, examining the process of fear learning is an important aspect in the attempt to prevent and treat PTSD. Understanding human fear learning would ideally involve the examination of these processes in the human brain, however, there are clear limitations to this capability. The rodent brain has been used extensively to gain insight into the cellular mechanisms underlying fear learning (73). Through rigorous experimentation in available rodent models, we can optimize our ability to gain understanding of the cellular processes taking place in the brain and subsequently begin to take measures toward prevention and more effective treatment. The ability to capture "at risk" individuals (those that exhibit enhanced or exaggerated fear response) could lead to pre-emptive steps aimed at reducing the incidence of PTSD. Likewise, identifying at risk individuals in the general population could lead to preventative measures in the workplace or as part of routine medical care in an effort to reduce the development of PTSD in the event of trauma.

PAVLOVIAN FEAR CONDITIONING AS A MODEL OF FEAR LEARNING

Pavlovian fear conditioning models a core feature of anxiety disorders and PTSD, namely memory formation between intrinsically fearful or unconditioned events and specific cues (34; 86). Fear responses can be evoked by stimuli that have gained emotional significance through classical conditioning (17; 45; 53). Classical (Pavlovian) conditioning of fear-provoking stimuli (fear conditioning) is inducible in all organisms studied to date (73). Fear conditioning requires the coordinated presentation or pairing of two sensory inputs (see Figure 1.4). One input is an initially neutral stimulus (which becomes the conditioned stimulus or CS), and the other input is a biologically salient

event (the unconditioned stimulus or US). The repeated pairing of these stimuli results in a conditioned response (CR) such that the initially neutral stimulus elicits the same behavioral response as the US. In the behavioral model of auditory fear conditioning in both humans and animals, a neutral auditory tone is paired with a mild electric shock. Repeated pairings of the stimuli result in associative learning such that the tone is associated with the shock and the tone alone begins to elicit a fear response (74). This model results in rapid learning that can last for a lifetime (108). As a result, Pavlovian fear conditioning is used by investigators as a reliable and powerful technique for investigating the cellular and molecular processes underlying emotional memory (45). The potential to identify effective strategies for the treatment of exaggerated fear memory that persists and leads to PTSD therefore, is aptly sought by employing fear conditioning experiments under controlled conditions (45).

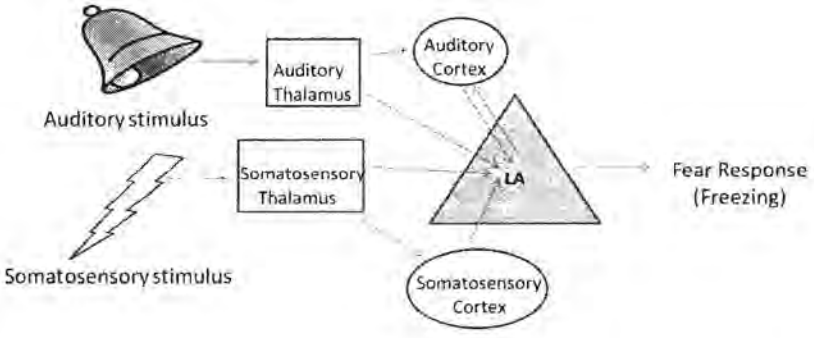


Figure 1.3. Schematic model of the neural circuitry of Pavlovian auditory fear conditioning. Model shows how an auditory conditioned stimulus and a nociceptive unconditioned foot shock stimulus converge in the lateral amygdala (LA) via auditory thalamus and cortex and somatosensory thalamus and cortex respectively. Thus the LA is a key site for associative memory formation and storage (adapted from LeDoux, 2004)

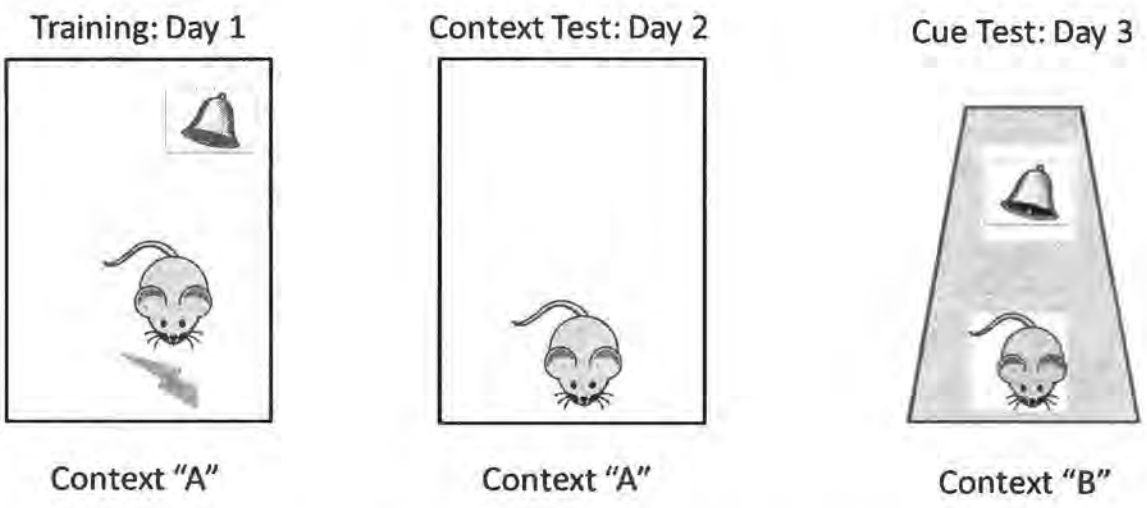


Figure 1.4. Schematic of Pavlovian fear conditioning. Following habituation to the conditioning chamber (context "A"), the animal undergoes training in that context. Training consists of the pairing of an auditory tone and a brief, mild electric footshock. The animal learns that the tone predicts the footshock. The following day, the animal is placed back into "context A" and fear to that context is measured by the amount of freezing seen by the animal during the testing period. No tones or shocks are administered. Fear specific to the tone is measured the following day in a novel context ("context B") when the tone is administered (without shocks). The novel environment does not elicit contextual fear, so freezing during the tone is specific to the auditory cue.

Pavlovian fear memory formation requires phosphorylated MAPK

A long-term fear memory is stored in the lateral amygdala and requires the phosphorylation of mitogen-activated protein kinase (pMAPK) (121). The phosphorylation of MAPK is triggered by synapse-activated calcium influx and leads to the initiation of new protein synthesis (16; 121). Inhibitors of pMAPK injected into the lateral amygdala have consistently resulted in fear memory consolidation deficits (44; 84; 119). Evidence suggests that formation of a long-term fear memory is a process of learning, and consequently, aspects of PTSD are suggested to result from maladaptive

learning (41). As pMAPK is a vital component in fear memory storage, its activation is an indicator or marker of the neural network underlying fear memory. Thus, pMAPK in the LA has become an effective measure of long-term fear memory formation and a means by which fear-related pathology is investigated at the cellular level (119; 123). This research will use the Pavlovian fear conditioned mouse model to examine behavioral differences, neuronal activity alterations, and indicators of cellular plasticity (pMAPK neurons) between high and low fear phenotype mice. The aim is to identify cellular mechanisms underlying high and low fear behavior resulting in high and low 'fear load'. High 'fear load' is said to exist in individuals with PTSD (89), and is therefore, an area of research interest.

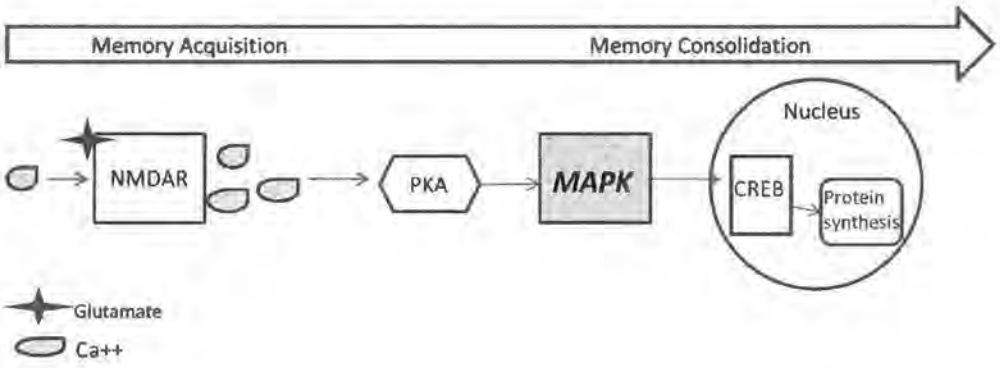


Figure 1.5. Memory consolidation requires phosphorylated MAPK (pMAPK). Excitatory receptor activation (e.g. NMDAR) stimulates the influx of calcium, activation of second messengers (e.g. PKA), phosphorylation (activation) of MAPK by MEK (MAP kinase kinase) an immediate precursor kinase to MAPK, activation of transcription factors (e.g. CREB), and mRNA synthesis leading to new proteins. Adapted from Lamprecht & LeDoux, 2004. (69)

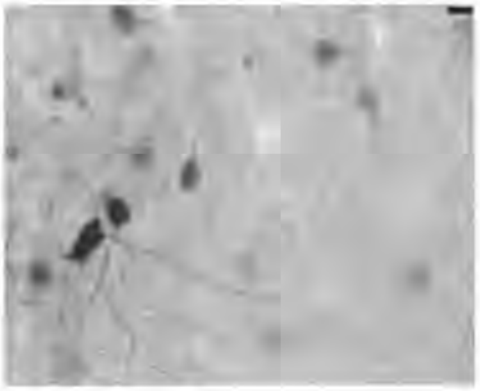
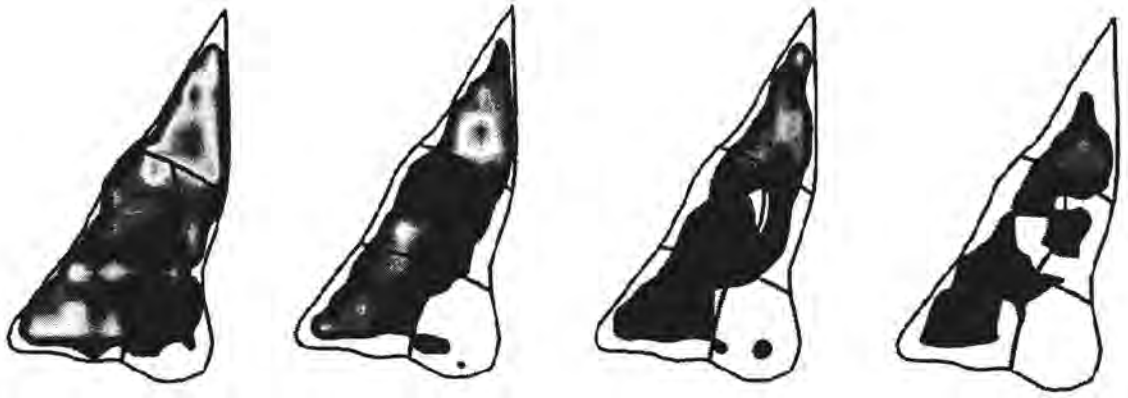


Figure 1.6. Photomicrograph of immunohistochemically-processed mouse brain (LA) section depicting pMAPK-expressing neurons at 20x objective. pMAPK is a known marker of learning-induced plasticity in the LA and is suggested to peak at 1 hour following fear memory acquisition. (Coyner *et al.* unpublished)

Pavlovian fear conditioning results in a consistent pattern of neuronal activation in the amygdala

Recent publications from our lab suggest that pMAPK expressing neurons following Pavlovian fear conditioning form a consistent spatial pattern. These data have been replicated in several experiments (13) (and unpublished data) and suggest that an associative fear memory trace (i.e. a fear memory that is linked specifically to a discrete sensory cue such as a tone) may have a unique topography within the amygdala (see Figure 1.7). One question we ask as a result of this finding is whether high fear phenotype mice have a different pMAPK expressing neuron topography compared to low fear phenotype mice. Identifying unique "hallmarks" of high and/or low fear could have important medical implications from which novel strategies for prevention and treatment may be developed.



fear conditioned unpaired tone/shock shock alone naive

Figure 1.7. A fear memory trace following Pavlovian fear conditioning in Sprague-Dawley rats shows evidence of a consistent spatial pattern of neuron activation (neurons expressing pMAPK). This pattern is unique in comparison to naïve and unpaired (non-associative fear learning) groups. Heat maps demonstrate pMAPK expressing neuron density (blue=low, red=high; mean per group) in coronal sections of the basolateral amygdala in four different experimental groups. Uppermost region (triangle-shaped)=Dorsolateral amygdala (LAd), middle left=ventromedial lateral amygdala (LAvm), middle right=ventrolateral amygdala (LAvl), lower left=basomedial nucleus (BM), lower right=basolateral nucleus (BL) Bergstrom *et al.* 2011.

MOUSE MODEL OF HIGH AND LOW PAVLOVIAN CONDITIONED FEAR

In order to attempt to determine genes that underlie high and low fear, two inbred strains of mice, C57BL/6J and DBA/J2 were used by Ponder *et al* (2007) to create a hybrid mouse line with distinct differences in fear conditioning (101). Tests of acute shock sensitivity and general learning ability revealed no underlying differences between the high and low fear lines, however divergence existed in fear learning and measures of anxiety (101). Beginning with the S1 generation, we repeated the breeding regimen used

by Ponder *et al* (2007), in order to conduct further experiments aimed at examining cellular mechanisms involved in high and low Pavlovian fear memory. We received High and Low line breeding pairs (S1 generation) of the advanced intercross line (AIL) that then underwent further selection for high and low fear memory. S1 High and Low line mice were fear conditioned (two paired tone/shocks: 75 decibel tone x 30 seconds co-terminated with a 0.5mA foot shock x 2 seconds) in "context A" and "high freezers" were separated from "low freezers". The following day, mice were returned to "context A" for ten (10) minutes and the duration of freezing (an index of fear in the rodent) was measured. The mean freezing score for the duration of context test was used to select high freezers from low freezers. Once selected, "high freezers" were bred together and "low freezers" were bred together. Only the extremes of freezing were selected for breeding which was approximately the highest and lowest 10-30% of those tested. Animals that scored in the middle range of freezing scores were not selected for breeding. Offspring from those breeding pairs underwent identical testing beginning at the age of 7 weeks. No sibling or cousin pairings were made. Documentation of each animal was maintained by the designation of "S" ("selected" according to freezing status) such that "S3" indicates the mice from the third generation of selected high and low freezing scores. A high degree of freezing suggests "High line" and a low degree of freezing suggests "Low line" animals. Use of this breeding regimen resulted in mice that exhibit distinct and divergent high and low fear memory that allow us to examine cellular mechanisms of fear phenotype (101). The S4 generation of High and Low line mice was used for experiments here. (see Figure 1.8 for breeding schematic).

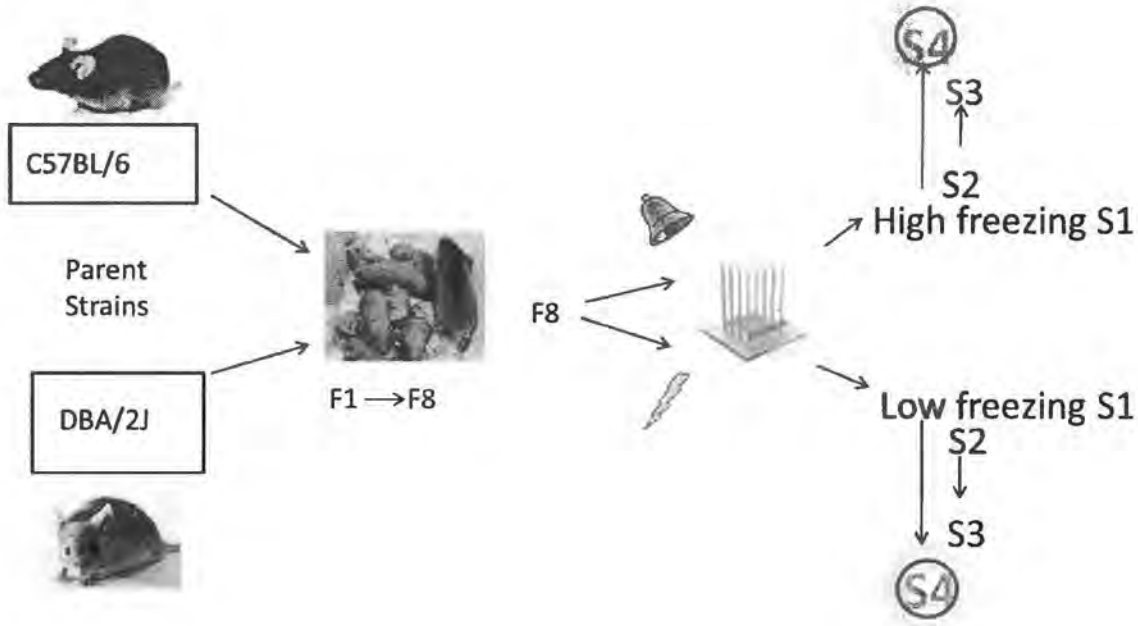


Figure 1.8. Schematic of breeding regimen used to obtain High and Low line mouse models. The S4 generation was used for experiments described. (Coyner, 2012 unpublished)

CHAPTER 2: Mice selectively bred for High and Low fear behavior show differences in the number of pMAPK (p44/42 ERK) expressing neurons in lateral amygdala following Pavlovian fear conditioning

ABSTRACT

Individual variability in the acquisition, consolidation and extinction of conditioned fear potentially contributes to the development of fear pathology including posttraumatic stress disorder (PTSD). Pavlovian fear conditioning is a key tool for the study of fundamental aspects of fear learning. Here, we used a selected mouse line of high and low Pavlovian conditioned fear created from an advanced intercrossed line (AIL) in order to begin to identify the cellular basis of phenotypic divergence in Pavlovian fear conditioning. We investigated whether phosphorylated MAPK (p44/42 ERK/MAPK), a protein kinase required in the amygdala for the acquisition and consolidation of Pavlovian fear memory, is differentially expressed following Pavlovian fear learning in the High and Low fear lines. We found that following Pavlovian auditory fear conditioning, High and Low line mice differ in the number of pMAPK-expressing neurons in the dorsal sub nucleus of the lateral amygdala (LAd). In contrast, this difference was not detected in the ventral medial (LAvm) or ventral lateral (LAvl) amygdala sub nuclei or in control animals.

We propose that this apparent increase in plasticity at a known locus of fear memory acquisition and consolidation relates to intrinsic differences between the two fear phenotypes. These data provide important insights into the micro network mechanisms encoding phenotypic differences in fear. Understanding the circuit level cellular and molecular mechanisms that underlie individual variability in fear learning is critical for the development of effective treatment of fear-related illnesses such as PTSD.

INTRODUCTION

Individual variability in the acquisition, consolidation and extinction of conditioned fear is linked to the pathophysiology of fear disorders including post traumatic stress disorder (PTSD) (26; 58; 89; 91). While the majority of people that experience traumatic events during their lifetime do not develop PTSD, a small percentage of individuals do (64). Pavlovian fear conditioning represents a key model for the study of processes related to PTSD (56; 58; 89). Fear-related pathology may develop in individuals with extreme phenotypes who are highly reactive to traumatic events and slow to recover during extinction of those events (89). On the contrary, resilience, or the ability to adapt to adversity, is suggested to be characterized by low reactivity and fast recovery during extinction (26; 137). As such, PTSD is associated not only with the severity of the trauma (i.e. increased trauma severity increases the likelihood of developing PTSD) but also with the severity of the *reaction* to trauma by an individual (24; 40) (i.e. those with more severe early symptom responses are more likely to develop PTSD). In support of this hypothesis, recent clinical data by Norrholm and colleagues (2011) suggest that persons with PTSD develop a higher 'fear load' in response to the acquisition and consolidation of a new conditioned fear memory (89).

The amygdala is directly implicated in PTSD. Evidence from clinical studies comparing individuals with PTSD to healthy controls shows that those with PTSD have increased amygdala activity to both negative stimuli and to trauma specific stimuli (104). The amygdala is a key brain structure in emotional processing and the various subnuclei that comprise the amygdala play a critical role in the acquisition, consolidation, and behavioral response to associative fear (99; 108). The lateral amygdala (LA) subdivides into three distinct regions called the dorsolateral (LAd), ventral medial (LAvm) and

ventral lateral (LAvl). While there are less data relating to the individual roles that these LA subregions play in fear processing, the LAd is proposed to be the primary locus of sensory and somatosensory synaptic convergence (111). The LAd projects to LAvl and LAvm but LAvl and LAvm do not appear to signal to each other or back to the LAd (99). Thus the LAd is located at the apex of an anatomical and functional network of LA subnuclei. Work in recent decades provide important insight into the cellular and molecular processes underlying the formation of enduring fear memories and Pavlovian fear conditioning has been an invaluable tool in such discoveries (43; 74; 106). This form of classical conditioning pairs a previously neutral cue such as a tone (conditioned stimulus or CS) with an aversive stimulus such as a foot shock (unconditioned stimulus or US). When presented so that one is temporally associated with the other (CS+US), the association is learned and future presentation of the CS alone will elicit a conditioned response (CR) identical to presentation of the US alone.

The LA is the key site for the convergence of sensory stimuli transmitting the CS and US (76; 113). Moreover, the LA is a site for the cellular changes underlying the acquisition and consolidation of Pavlovian fear (74; 79; 93). Memory consolidation and maintenance is the result of plastic changes involving excitatory synaptic transmission and intracellular signaling leading to new protein synthesis (8; 32; 69; 108). Phosphorylated mitogen-activated protein kinase (p44/42 ERK/pMAPK) is required in the LA for the long-term (but not short-term) storage of an associative fear memory through stabilization of long-term potentiation (LTP) from early to the late phase LTP (108; 118). The MAPK cascade involves a series of kinases that lead to downstream activation of transcription factors (such as CREB) and subsequent new protein synthesis

(35; 122). This signaling pathway has diverse functions including cell proliferation and differentiation (122), however in the amygdala this pathway is necessary for the long-term consolidation of an associative fear memory (35; 108). In the LA, pMAPK expression is induced in a select population of neurons in response to Pavlovian fear learning (12; 13; 58; 119). Presentations of the CS or US alone or in a temporally non-paired manner do not result in a Pavlovian fear conditioned memory or in a significant increase in pMAPK neurons (12; 13; 58; 119).

What makes individuals differently susceptible to Pavlovian fear conditioning is an important question for helping to understand individual susceptibility to some anxiety disorders including PTSD. In order to begin to answer this question, it is necessary to understand the cellular basis of divergent Pavlovian fear phenotypes. We began with an F_8 generation C57BL/6J x DBA/2J (B6D2) AIL and selected over three generations to establish divergent mouse lines with a genetic disposition to high and low fear learning after Pavlovian conditioning (94). Mice were selected for high or low contextual and cued Pavlovian fear conditioning (58; 94; 101). Offspring of mice selected for high and low fear (Highs and Lows respectively) were used for parallel behavioral and cellular comparison. To ascertain whether differences in the number of neurons expressing pMAPK correspond to the divergent associative fear learning, we quantified pMAPK neuron numbers in the LA following the induction of Pavlovian fear conditioning in Highs and Lows. This initial examination of whether differential plasticity as measured by pMAPK expressing neurons exists in the LA of divergent lines at baseline and following Pavlovian fear conditioning should be followed by examination of other brain

regions within the fear processing circuitry in order to fully elucidate the mechanisms of divergent fear memory.

MATERIALS AND METHODS

Animals

High and Low Pavlovian fear mouse lines

Short-term selection for contextual fear was used to create outbred mouse lines with robust differences in fear learning (101). Mice were phenotyped and selected beginning with the F_8 generation C57BL/6J x DBA/2J AIL (B6D2 F_8) obtained from the University of Chicago (94). The mice that exhibited the highest and lowest contextual freezing one day following Pavlovian fear conditioning were selected to create new breeding pairs in the High and Low lines, respectively (10-30% each of the High and Low populations to create 12 breeding pairs in each line). Freezing to the CS was used as an additional measure for selection when necessary. Siblings and first cousins were never paired for breeding. Offspring were fear conditioned at 8-10 weeks of age, selected for high and low contextual fear and High and Low line breeding pairs were again formed. This process continued for 3 selection generations (S1-3). Behaviorally naïve, adult male mice from the 4^{th} selected generation were used for experiments described here. Mice were randomly assigned to one of two parallel experimental cohorts: behavior (BEH), or immunohistochemistry (IHC) and were then further randomly assigned to one of three groups: Naïve (to US and CS), Tone (CS alone), or Paired (Conditioned, US and CS).

Husbandry

Mice were housed 2-5 per cage segregated by sex and line (High or Low) in standard shoebox cages in a climate controlled vivarium on a standard 12hr light/dark

cycle with ad libitum food and water. Experiments were conducted during the light cycle. All experiments adhered to IACUC approved protocols and procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals.*

Pavlovian fear conditioning

Forty-eight adult male mice aged 2-8 months were used for all behavior experiments. Conditioning chambers (Coulbourn Instruments, Whitehall, PA) measured 7"Wx7"Dx12"H and were inside sound attenuation chambers. Two distinct environments were created with these chambers in order to represent box "A" and box "B". Alterations to the environments involved changes in flooring (grid shock floor for training and context test; wire mesh non-shock floor for cue test), chamber dimensions and appearance, lighting, and scent (70% isopropyl alcohol cleaning solution versus commercial cleanser). Mice were transported to a holding area free of high human traffic and noise and they remained in this area for 30 minutes prior to being placed into the conditioning chambers. Behavior experiments consisted of 3 days of habituation to "A", training day in "A", context test day in "A", and cue test day in "B" (6 consecutive days, see figure 1).

Pavlovian fear conditioning consisted of three paired tone/shock presentations (CS/tone+US/foot shock x3) over 10 minutes with approximately 1-2 minutes between CS+US pairings (the inter-trial interval or ITI). Tones were 75dB, 5000Hz and lasted 30 seconds. Shocks were 0.6mA for the final 1 second of the tone. Animals were allowed 3 minutes in the chamber prior to any stimuli and 2 minutes elapsed following the final tone/shock pairing prior to removal from the chamber. Mice assigned to the fear

conditioning group receiving both CS and US are referred to as "Paired" (n= 12 Highs and 12 Lows). Mice assigned to the "Tone" groups (CS only) received training identical to the paired groups, however no shocks were administered (n= 6 Highs and 6 Lows). "Naïve" animals were placed in the conditioning chambers for equivalent periods of time with no CS or US (n= 6 Highs and 6 Lows). One day following fear conditioning, animals were returned to the training context "A" for 10 minutes and freezing was scored. The next day (two days following training), in order to isolate associative cued fear, mice were placed into a novel environment "B" and three tones identical to the training CS in quality and duration were administered over a 10-minute period of time. Freezing, an active behavior, is the cessation of all movement except for movements associated with respiration (18). Freezing was measured using FreezeFrame™ (Coulbourn Instruments, Whitehall, PA) automated scoring and verified by the investigator. Adjustments to threshold were made so as to accurately detect freezing and varied depending on mouse coat color (gray, light brown, or black) and lighting in each chamber.

Scoring of freezing

Freezing was measured at time-matched points across groups as follows: Training Day: during the thirty seconds of tone+/- shock 2 and 3 (or equivalent time points for Naive mice), Context Test Day: during the initial 5 minutes in the chamber, and Cue Test Day: during tone 1, 2, and 3 with baseline novel context freezing subtracted from tone 1 freezing. All data are means per group.

Immunohistochemistry (IHC)

Mice underwent 3 days of habituation to context A followed by training as described in behavior experiments. One hour following training, mice in the IHC cohorts were anesthetized with ketamine/xylazine (100mg/kg + 10mg/kg) via intraperitoneal injection and transcardially perfused (Gravity Perfusion System for mouse, AutoMate Scientific, Berkeley, CA) with 20ml 0.9% normal saline followed by 40ml 4% paraformaldehyde (FD Neurotechnologies, Columbia, MD). Brains were post-fixed overnight and transferred to 1X phosphate buffered saline (PBS) until processing for immunohistochemistry. Brains were sliced on a vibratome at 40μm and marked in a consistent manner to indicate rostro-caudal sequence. Free-floating sections (5 per well) were placed in 1% BSA blocking solution for one hour prior to incubation in rabbit polyclonal primary antibody to phospho-p44/42 MAPK (1:250 dilution, Cell Signaling Technology, Boston, MA) for 24 hours at room temperature. Following 5 washes in PBS, sections were incubated in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200 dilution, Vector Laboratories) for 30-minutes, washed x4, and then placed in avidin-biotin HRP complex (ABC Elite, Vector Laboratories) for 1-hour. Visualization of pMAPK-expressing neurons was achieved by SG chromagen/hydrogen peroxide (Vector Laboratories). Sections were mounted in sequence on Superfrost ™ slides, allowed to completely dry, and then dehydrated in increasing percentages of alcohol (50%x1, 70%x1, 95%x1, 100%x2) followed by xylene.

Section Alignment

Analysis of pMAPK-expressing neurons was conducted on matched amygdala slices across subjects. Matching sections across subjects was achieved by using

consistent, identifiable landmarks and the Franklin and Paxinos "The Mouse Brain in Stereotaxic Coordinates" (46) (see figure 3). The optic tract reliably appears and lengthens in coronal sections placed in rostral to caudal sequence. We matched sections across subjects by using the relationship between the right optic tract and the central nucleus (CeA) of the amygdala (see Figure 3). Sections in this analysis were from bregma -1.58mm, -1.70mm, and -1.82mm. Contours of the lateral amygdala (LA) were traced for each bregma coordinate and applied to each section so as to count from a consistent area that accurately depicts the region of interest as illustrated in the mouse brain atlas.

Statistical Analysis

Data were analyzed using GraphPad Prism (version 5) statistical software. Unless otherwise stated, two-way analysis of variance (ANOVA) with post-hoc t-test was used for each analysis. Data are expressed as mean +/- Standard Error of the Mean (SEM) and significance is defined as $p<0.05$.

RESULTS

Behavioral results

In the parallel behavioral group both Highs and Lows acquired Pavlovian fear but show differences in the strength of Pavlovian fear acquisition. Measurement of freezing to tone 2 and 3 during the acquisition of Pavlovian fear conditioning ("training", Day 1) revealed that both Highs and Lows acquired Pavlovian fear (figure 2A). A significant interaction between phenotype and experimental group exists [$F(2, 52) = 9.79$, $p = 0.0002$]. Lows in the Naive and Tone groups did not differ in freezing (mean 1.54 +/- 0.355 % and 3.17 +/- 1.985 %, $p = 0.438$). However, Lows in the Paired group exhibited

significantly more freezing compared to controls (mean 13.18 +/- 2.39%, $p = 0.0019$). Highs demonstrated a similar response to training across experimental groups. Naive and Tone Highs did not differ in freezing (mean 3.11 +/- 1.265% and 9.04 +/- 3.868%, $p = 0.175$). However, Paired Highs exhibited significantly more freezing (mean 47.97 +/- 4.10%, $p < 0.0001$). Importantly, comparison of control groups revealed no difference between Highs and Lows (Naive Highs vs. Naive Lows $p=0.258$; Tone Highs vs. Tone Lows $p = 0.206$). Freezing in Paired Highs was greater than Paired Lows ($p <0.0001$). Both phenotypes freeze during the tone once it has been paired with a foot shock. Thus, at training both Paired Highs and Paired Lows acquire Pavlovian fear memory and Paired Highs demonstrate greater freezing during this acquisition.

High fear mice freeze more upon return to training context A 1 day after training (figure 2B). Measurement of freezing during testing of consolidated contextual Pavlovian fear memory ("context test", Day 2) revealed that both Paired Highs and Paired Lows consolidated a contextual fear memory. However, a difference was seen between the Highs and Lows in the strength of the contextual fear memory. A significant interaction between phenotype and experimental group exists [$F (2, 41) = 3.29$, $p= 0.047$]. Lows in the Naive and Tone groups did not exhibit a difference in freezing (mean 0.818 +/- 0.610%, and 0.376 +/- 0.257%, $p= 0.510$). However, Lows in the Paired group exhibited significantly more freezing compared to controls (mean 12.21 +/- 3.35%, $p= 0.0106$). High Naive and Tone groups did not differ in freezing (mean 0.506 +/- 0.146, and 0.492 +/- 0.244%, $p= 0.962$). However, Paired Highs exhibited significantly more freezing than Highs in control groups (mean 40.30 +/- 8.41, $p= 0.0020$). Comparison of control groups revealed no difference between Highs and Lows (Naive Highs vs. Naive Lows: p=

0.661, Tone Highs vs. Tone Lows: p= 0.755). Importantly, data revealed a difference between Paired Highs and Paired Lows (p= 0.0052). Thus, context test data suggest that both Paired Highs and Paired Lows consolidate a Pavlovian contextual fear memory but that Paired Highs appear to consolidate a stronger contextual fear memory.

High fear mice freeze more upon presentation of the fear-associated cue (figure 2C). Isolating fear to the specific cue was assessed by placing mice in a novel context (B) two days following training and re-administering the CS. Measurement of freezing during testing of consolidated cued Pavlovian fear memory ("cue test", Day 3) revealed that both Paired Highs and Paired Lows consolidated a Pavlovian cued fear memory. However, a difference was seen between the Highs and Lows in the strength of the cued fear memory. A significant interaction between phenotype and experimental group exists [F (2, 42) = 3.65, p= 0.035]. Lows in the Naive and Tone groups did not exhibit a difference in freezing (mean 13.82 +/- 6.80%, and 5.54 +/- 3.75%, p= 0.311). However, Lows in the Paired group exhibited significantly more freezing compared to controls (mean 29.76 +/- 5.65%, p= 0.0224). High Naive and Tone groups did not differ in freezing (mean 14.44 +/- 5.44, and 7.06 +/- 5.87%, p= 0.237). However, Paired Highs exhibited significantly more freezing than Highs in control groups (mean 60.62 +/- 6.28, p<0.0001). Comparison of control groups revealed no difference between Highs and Lows (Naive Highs vs. Naive Lows: p= 0.944, Tone Highs vs. Tone Lows: p= 0.734). As in training and context test, cue test data revealed a difference between Paired Highs and Paired Lows (p= 0.0014). Thus, cue test data suggest that while both Paired Highs and Lows consolidated a cued Pavlovian fear memory, Paired Highs consolidate a stronger

fear memory to the cue. In a novel context, presentation of the fear-associated cue resulted in twice as much freezing in the Paired Highs compared to Paired Lows.

Immunochemistry results

Paired mice had more pMAPK-expressing neurons in the LAd compared to control mice and among those, Paired Highs had more pMAPK-expressing neurons than Paired Lows (figure 4A). We quantified pMAPK-expressing neurons in the LA in three matched coronal sections (bregma -1.58mm, bregma -1.70mm, and bregma - 1.82mm) according to the Franklin and Paxinos "The Mouse Brain in Stereotaxic Coordinates" (3rd ed., 2008). All subjects across groups and phenotype had pMAPK-expressing neurons in the LA one-hour following training. A difference in pMAPK expressing neuron number was seen in Paired Highs and Paired Lows in the LAd (two-way ANOVA: [$F(2, 30) = 4.05$, $p = 0.0277$]. Lows in the Naive and Tone group did not exhibit a difference in pMAPK-expressing neurons (mean 11.53 +/- 2.070% and 18.33 +/- 2.62%, $p = 0.0690$). However, Lows in the Paired group exhibited significantly more pMAPK neurons compared to controls (mean 27.73 +/- 2.52, $p = 0.0010$). Naive and Tone Highs did not differ in pMAPK neuron number (mean 16.00 +/- 2.77% and 21.33 +/- 3.94%, $p = 0.294$). However, Paired Highs exhibited significantly more pMAPK neurons (mean 50.26 +/- 6.85%, $p = 0.0003$). Comparison of control groups revealed no difference in pMAPK neuron numbers between Highs and Lows (Naive Highs vs. Naive Lows $p=0.225$; Tone Highs vs. Tone Lows $p = 0.5406$). Importantly, data revealed a difference in pMAPK neuron number between Paired Highs and Paired Lows ($p = 0.0115$). Quantification of pMAPK-expressing neurons in the LAvm did not reveal a difference among experimental group or phenotype [$F(2, 30) = 0.84$, $p = 0.441$], [F (2,

30) = 1.62, p = 0.213] (figure 4B). Quantification of pMAPK-expressing neurons in the LAvl did not reveal a difference among experimental group or phenotype [F (2, 30) = 1.40, p = 0.260], [F (2, 30) = 0.76, p = 0.389] (figure 4C).

DISCUSSION

We used a B6D2F$_8$ advanced intercross (C57B6/6J and DBA/2J) mouse line selected over 3 generations for differential expression of contextual and cued Pavlovian fear (101). We investigated endogenous differences in cellular plasticity in the lateral amygdala between the phenotypes. We determined that pMAPK, a protein required for the consolidation of Pavlovian fear memory, is differentially expressed in the LA of mice expressing divergent Pavlovian conditioned fear. Systematic matching of the rostral to caudal location of amygdala containing brain sections allowed for quantitative measurement of pMAPK expressing neuron numbers within the three sub nuclei of the LA (LAd, LAvl, LAvm). No differences in pMAPK neuron number were found in either the LAvm or the LAvl between the control and paired (Pavlovian fear) groups or between High and Low fear phenotypes. In contrast, a significant difference in pMAPK expressing neuron number was found in the LAd both between control and paired group, and also between the High and Low fear phenotypes. Thus, a difference in pMAPK expression mediated by different numbers of neurons expressing pMAPK is associated with a genetically selected differential expression of Pavlovian fear. These data provide important insights into the micro network mechanisms encoding both normal and potentially pathological fear.

The advantage of using an AIL over comparisons of inbred mouse strains differing in fear memory behavior is a that a tighter association of genes and traits can be

made from the highly recombinant AILs (10; 92; 94; 101). Selected animal lines developed from an AIL allow for increased specificity in isolating genetically determined behavioral and physical phenotypes due to a breakdown in linkage disequilibrium (58; 92; 94). Traits unassociated with the behavior of interest remain evenly distributed between the lines while relevant traits will segregate with the behavior (92; 94). Previous studies in the B6D2 AIL identified several narrow chromosomal regions and possible genes underlying the behavioral responses (92). The reduction of behavioral differences between the selected lines to fear and anxiety related behaviors is intended to isolate cellular mechanisms that drive the phenotypes and make them more likely to be identifiable (58; 92; 94).

AIL alleles originate in the founder strains. An early study by Paylor and colleagues (1994) (95) suggested that the DBA/2J (D2) strain has a hippocampal deficit when compared against the C57BL/6J (B6) strain. They found impaired hippocampal dependent contextual fear conditioning and no difference in amygdala dependent cued fear conditioning. A more recent study by Balogh and colleagues (9) confirmed that the D2 show less freezing to the same contextual shock paradigm compared to B6. In addition they showed that B6 show more contextual fear generalization and poor discrimination of contexts compared to the D2. These two studies (9; 95) show the fundamental differences in fear behavior between the strains. In our B6D2 F_8 AIL mice it is possible that aspects of these D2 characteristics are transmitted to the Low fear mice whereas the B6 characteristic are transmitted to the high fear mice. If this is the case a key question is which mechanisms within the network are modified by the transmitted genes (56; 74).

Importantly, the 8 generation AIL has extensive allele fragmentation (94). Thus the intercrossed mice and the subsequent segregated lines contain alleles transmitted from the foundation lines but likely contain novel allele combinations. These novel combinations may result in novel phenotypes. Importantly we observe that our High and Low fear lines show different levels of both contextual and cued fear (Figure 2) unlike the B6 and D2 which differ only on contextual fear (9; 95). The foundation strain also shows other behavioral and physiological differences possibly contributing to the novel High and Low fear B6D2 line. Moreover, differences in fear responses between the B6 and D2 founding strains include both freezing and autonomic responding. Steidl and colleagues (128) compared both C57BL/6J (6J) and 6N (6N) with the DBA/2J (2J) and the 2N (2N). They identified that not only do the 2J and 2N show less freezing than the 6J and 6N they also showed reduced heart rate activation compared to the 6J and 6N. These data suggest that the differences in fear are likely centrally mediated by amygdala. Amygdala afferents regulate both freezing and autonomic responses (56; 74). These data are important because they also point to the amygdala as a key site in the selected lines for physiological manifestations of genetic differences transmitted by B6 and D2 founding strains.

Little is known about the cellular and molecular mechanisms that underlie high and low fear response and yet, the underpinnings of these divergent responses must be understood in order to effectively treat fear-related illness (58). Data from these experiments indicate that High fear mice have more pMAPK-expressing neurons in the LAd following Pavlovian fear conditioning (figure 4). First, High fear mice potentially have a more dense fear network involving more neurons within the lateral amygdala fear

circuit. If this is the case, the increase in pMAPK expressing neurons observed may be a function of increased total neuron population. A second possibility is that the actual neuron numbers are equivalent between the High and Low fear lines, but that amygdala afferent synaptic strength is increased leading to more pMAPK expression per neuron. A third possibility is that there are differences in upstream effectors of the MAPK signaling cascade such as MEK (MAPK kinase) and Raf (MEK kinase) leading to differences in efficiency or sensitivity of the MAPK cascade between High and Low animals (35; 122). Finally, in LA neurons the MAPK cascade is induced through neuronal calcium influx and depolarization (108). Differences between High and Low animals in the number of pMAPK neurons may therefore reflect functional differences in membrane receptors or ion channels of LAd neurons between High and Low lines.

Differences in pMAPK expressing neuron numbers between High and Low line mice were specific to the LAd. The anatomical specificity of these differences further supports the role of the LAd as the apex in a pathway of plasticity within the amygdala following Pavlovian fear conditioning. Repa and colleagues (2001) identified two different populations of neurons exhibiting plasticity within the LAd following Pavlovian fear. First, a population of neurons in the dorsal tip of the LAd exhibited transiently plastic neurons. In contrast, a second population was identified in the ventral LAd that exhibited long term plasticity. These differences may have been driven by differences in afferent connectivity with the thalamus targeting the short-term plastic neurons while cortical and intra-amygdala projections may target the ventral long term plastic LAd neurons (105). We previously identified an intra-LAd circuit that supports a role for feedback-mediated plasticity within the LAd (54; 57). While plastic changes have also

been identified in the LAvm and LAvl (12), studies of LA plasticity and connectivity suggest the LAd is the key site for initiation and storage of amygdala dependent plasticity. We recently showed in a rat model that Pavlovian fear conditioning is precisely localized to a population of neurons within discrete and stable micro regions within the LAd (12; 13). Importantly anatomical studies suggest that connectivity and communication among the LA subdivisions is unidirectional from LAd to LAvm and LAvl. Further no signaling is reported between LAvm and LAvl (100). Projections from within the amygdala to the LA terminate in LAvm and LAvl (100). Projections from the LA to the amygdala predominately originate in the LAvm (100). These functional and anatomical studies suggest that the LA sub divisions have a unique role in storage and processing of Pavlovian fear memories with the LAd at the apex of this circuit.

The finding that differences in pMAPK expressing neuron number are precisely localized to an LA sub nucleus in High and Low line mice suggests precise micro network differences between the two fear phenotypes. The approximate percentage of glutamatergic and GABAergic neurons in the LA in rat is 85% and 15% respectively (110). The total numbers of pMAPK expressing cells in the LAd occurs in approximately the same ratio (87% of pMAPK+ cells co-express CAMKIIa) (12). While these data in rat have not yet been confirmed in mice, it seems likely that, as in rat, the majority of fear learning induced plasticity in the LA is occurring in excitatory neurons. Following associative fear learning the High selected line mice have more pMAPK expressing neurons in the dorsolateral amygdala (LAd) compared to the Low selected lines. This experience-induced plasticity occurs within a key region of interest within the LAd (12; 13; 54; 56; 57; 105) where fear-associated auditory and somatosensory information

converge within the amygdala (52; 57; 76; 113). Mice in control groups show equivalent numbers of pMAPK expressing neurons and demonstrate that the increase in the pMAPK cell population is related to the learned CS-US association (figure 4). While these data alone are insufficient to establish this as a mechanism for increased fear memory, the data are compelling and provide important information for future experiments.

The mechanisms driving the differences in pMAPK neuron numbers are an important target for future research. Upstream regulators of calcium signaling, particularly AMPA receptors (116), are key targets for future research in these mice and similar models. Benedetto et al 2009 have proposed the use of pMAPK targeted drug therapy for use in anxiety disorders based on identification of the essential nature of ERK/pMAPK in both the consolidation and reconsolidation of fear memory (12; 13; 39; 58; 118). The present data showing that pMAPK expression increases after Pavlovian fear learning, also demonstrates the differential role of pMAPK in the relative strength of Pavlovian fear in a genetically heterogeneous population. The AIL fear phenotype mice are thus a useful model for testing ERK/MAPK-targeted pharmacotherapy for treatment of Pavlovian based aspects of fear pathologies (38; 58). An important follow-up experiment will be to pharmacologically inhibit pMAPK in both lines prior to fear conditioning and examine the behavioral and histochemical effects within and between High and Low line mice. Additionally, investigation of pMAPK-expressing neurons in other brain regions within the fear circuit such as BA, Ce, auditory thalamus, hippocampus, and prefrontal cortex may reveal other important differences between the lines that explain the divergent fear memory behavior.

The diagnosis of PTSD requires a set of criteria be met including experience of threat to life or the witnessing of this in others, prolonged hypervigilance, avoidance of reminders of trauma, and intrusive thoughts about the experience (6). While much is understood about the symptomatology of PTSD, little is known about the neurobiological mechanisms that underlie this devastating illness. It is unclear whether an increased conditioned response to fear stimuli is a consequence of or a predictor of PTSD (96). Recent prospective studies indicate conditioned fear may be a predictor of Post-Traumatic Stress Syndrome (PTSS) in firefighters (91). Some people may have pre-trauma vulnerabilities making them susceptible to posttraumatic stress and potentially more likely to develop PTSD (91). These data for PTSD support a body of evidence that stronger acquisition of conditioned fear is common to anxiety disorders (77). However, processes at the cellular and molecular level underlying the variable responses that lead to high reactivity and subsequent fear pathology are unknown. Therefore, identification of the neurobiological mechanism underlying differences in the acquisition of conditioned fear will aid the understanding of normal and pathological fear (26; 55; 89; 91).

Recent clinical data suggest that persons with PTSD develop a higher 'fear load' in response to the acquisition and consolidation of a new conditioned fear memory (89). Importantly, PTSD may thus be associated less with the severity of the trauma, and more with the severity of the reaction to trauma by an individual (24; 40). Individuals that exhibit high fear response during acquisition of a fear memory as well as when exposed to contextual and cued reminders of the fear experience may be the 'at risk' population for future development of PTSD. Mouse models of high and low Pavlovian conditioned

fear are therefore an important tool in the further discovery of mechanistic differences between individual variability in fear response and more efficacious treatment of fear-related illness.

In summary, these data provide important biological data on the cellular mechanisms of differences in conditioned fear between High and Low fear mice. Non-human animal models offer valuable insight into understanding the neurobiological mechanisms that underlie individual variability in acquisition and consolidation of fear memory (37). Because fear circuitry is conserved across species, the cellular and molecular mechanisms of human fear can be identified using non-human animals such as rodents (74). Selectively bred animal models that isolate distinct behavioral phenotypes such as very high or very low fear are a valuable tool to the study of how mammals learn fear. Further, examining extremes in fear behavior can better suggest mechanisms and lead to more effective treatment of fear-related illness in humans. Data from the present study may help provide important insights into individual differences in Pavlovian fear and how differences in pathological fear are established (58).

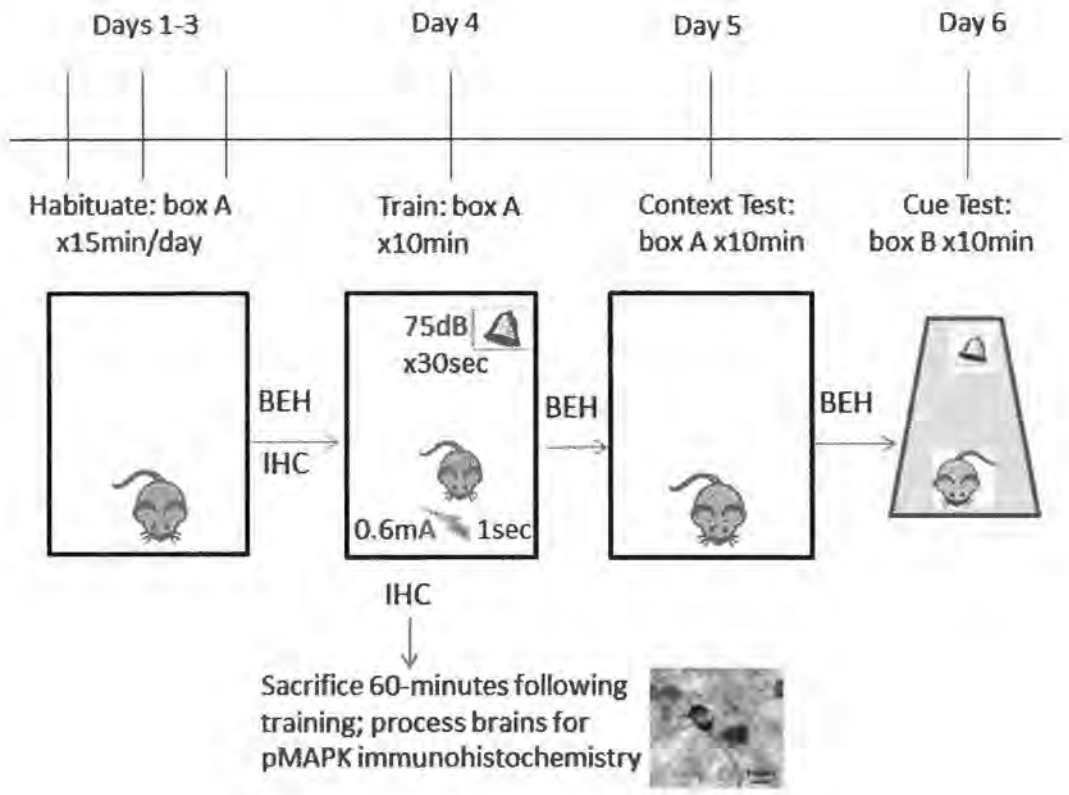


Figure 2.1. Experimental design for comparison of phenotypes in response to Pavlovian fear conditioning and analysis of pMAPK expression. Mice habituate to the conditioning chamber (A) for 15-minutes a day for three consecutive days immediately preceding training. All cohorts (Naïve, Tone alone, and Paired) undergo habituation to box A. On day four, mice receive training as indicated by random group assignment. Paired group animals receive 3 tone/shock pairings, Tone group animals receive identical training to the paired group without shocks. Naïve animals spend the same amount of time in the box with no tones or shocks. Subjects assigned to IHC are sacrificed 60-minutes following training and brains are subsequently processed to detect pMAPK-expressing neurons. One day after training, BEH subjects are returned to the training context (A) for context testing. On the final day of behavior experiments, subjects undergo cue testing during which they are placed in a novel context (B) and administered three tones identical in duration and quality to the training CS.

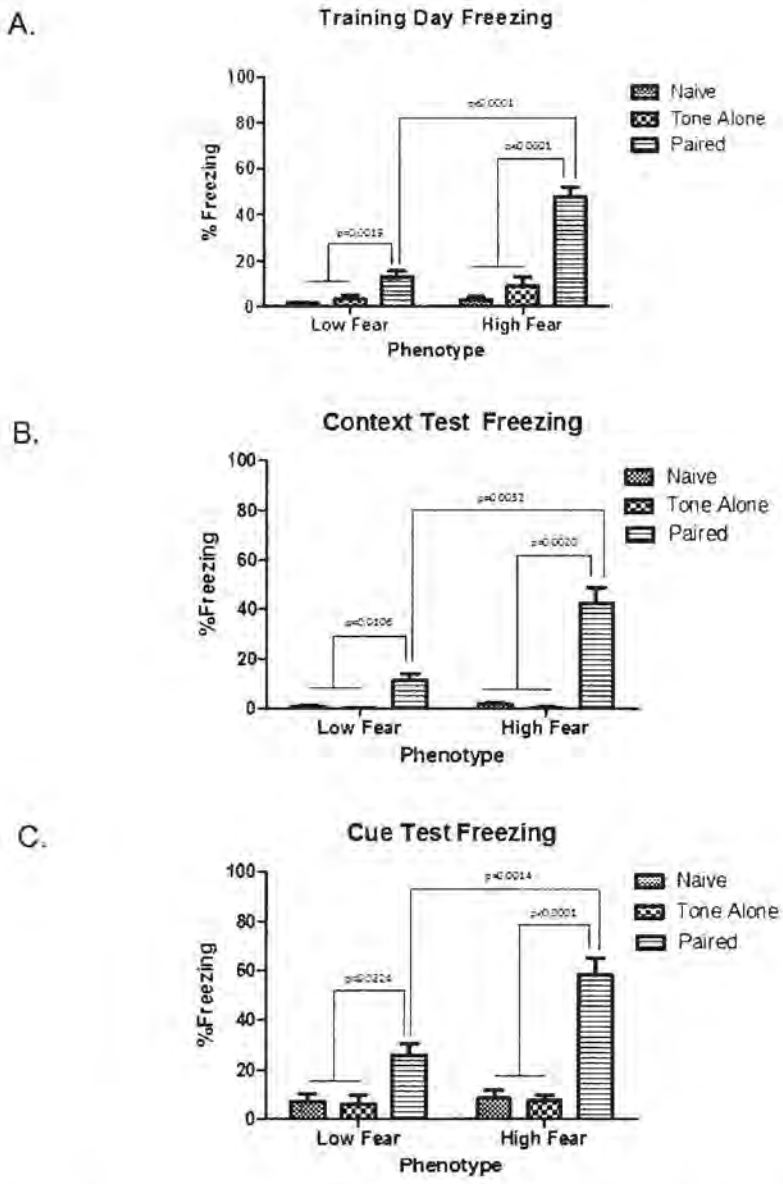


Figure 2.2. High and Low Pavlovian fear mice show differences in fear acquisition, context test, and cue test. (A) Training Day: High fear mice exhibit greater freezing relative to Low fear mice that receive identical training ($p<0.0001$). Low and High mice that received tone/shock pairings freeze more than control groups. Control groups do not differ. (B) Context Test Day: Both Low fear and High fear mice exhibit fear to the context. Fear conditioned High mice freeze more than fear conditioned Low mice suggesting stronger contextual fear memory consolidation. Tone group High mice exhibited less freezing than High naïve mice but differences are not significant (C) Cue Test Day: High mice exhibit greater freezing to the fear-associated auditory cue relative to Low mice.

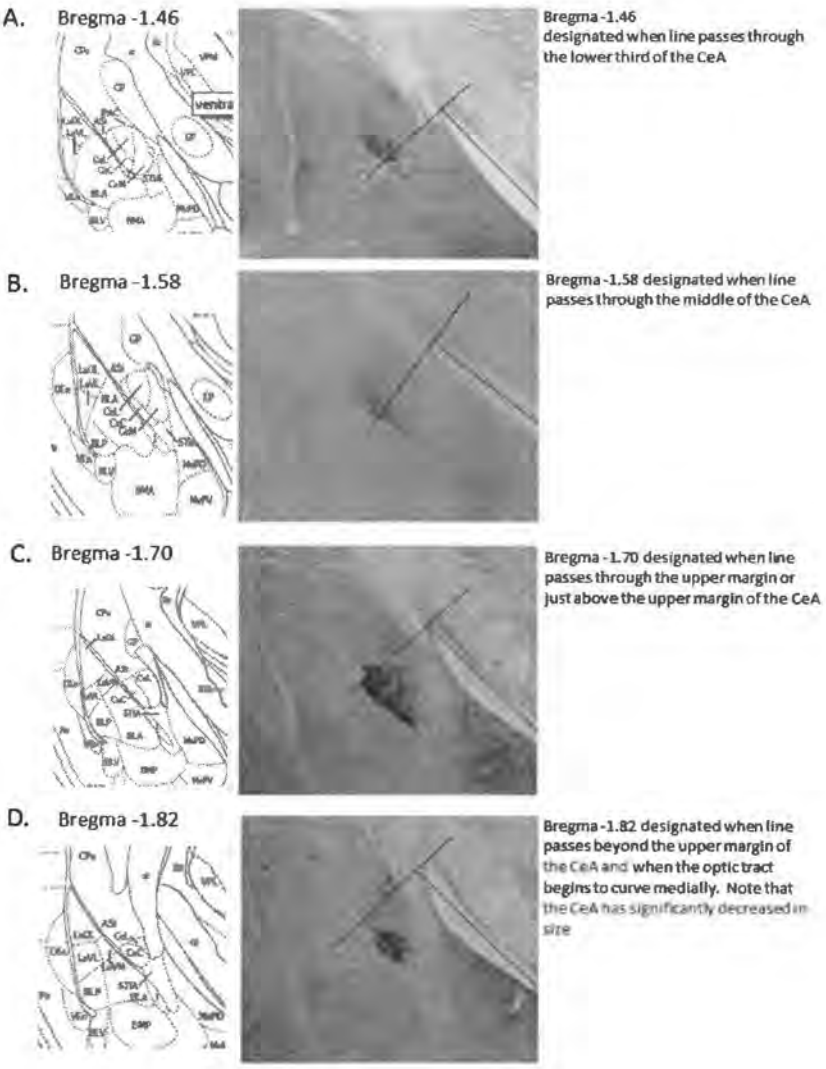


Figure 2.3. Anatomical demarcation of rostro-caudal location of lateral amygdala brain slices for pMAPK immunohistochemistry. Slices were compared to the Franklin and Paxinos mouse brain atlas (2008 at left, used with permission) for verification of the right central nucleus (CeA) in relation to the right optic tract. (A) Bregma -1.46 slices were designated when the right optic tract terminated at the lower third of the CeA and when the CeA was round in shape (not used for neuron quantification). (B) Bregma -1.58 slices were designated when the CeA appeared as a teardrop shape and the optic tract terminated at approximately the middle of the CeA. (C) Bregma -1.70 slices were designated when the optic tract was level with or extended just beyond the CeA but prior to when the optic tract curved medially. (D) Bregma -1.82 slices were designated when the optic tract extended beyond the superior border of the CeA and when it curved medially while retaining specific margins.

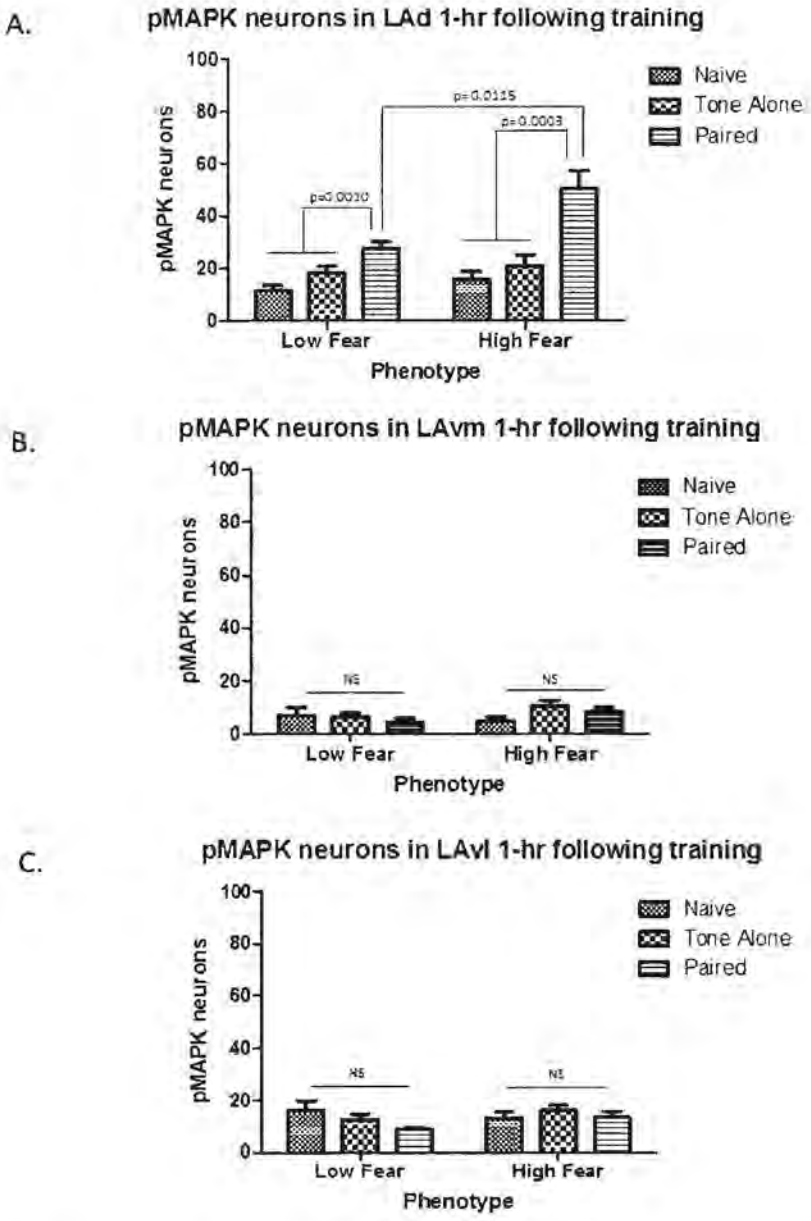


Figure 2.4. Following Pavlovian fear conditioning, High and Low fear mice have different numbers of pMAPK-expressing neurons in the LAd. (A) Total pMAPK-expressing neurons counted in the LAd in Highs and Lows in each experimental group. (B) Total pMAPK-expressing neurons counted in the LAvm in Highs and Lows in each experimental group. (C) Total pMAPK-expressing neurons counted in the LAvl in Highs and Lows in each experimental group. pMAPK-expressing neurons were quantified in the LA at 40x objective.

CHAPTER 3: The MEK Inhibitor SL327 differentially inhibits contextual and cued fear memory strength in mice selectively bred for high and low fear

ABSTRACT

Pavlovian fear conditioning is a widely used amygdala-dependent model of fear memory. However, the neurobiological mechanisms underlying fear memory strength are less well understood. Understanding the neurobiological mechanisms of fear memory strength is important in order to better understand disorders of fear memory including Post traumatic stress disorder (PTSD). PTSD is a serious medical condition affecting both military and civilian populations. While its etiology remains poorly understood PTSD is characterized by high and prolonged levels of fear response. Here, we used a selected mouse line of High and Low Pavlovian conditioned fear to begin to identify aspects of the cellular basis of phenotypic divergence in fear memory strength. We have previously demonstrated differences in the number of phosphorylated MAPK/ ERK (pMAPK) expressing neurons in the dorso-lateral amygdala (LAd) of these High and Low fear mice. Here we used a selective MEK inhibitor (SL327) to pharmacologically inhibit pMAPK/ERK prior to fear conditioning and examined fear memory strength and the quantity of pMAPK-expressing neurons in the LAd. We found contextual fear was abolished in both High Fear and Low Fear mice. In High fear mice, while contextual fear is completely abolished by SL327, cued fear was only reduced to ~50% of its control. In High fear mice we found SL327 reduced the number of neurons expressing pMAPK/ERK in the LAd. These data suggest that contextual fear is more sensitive to disruption by the MEK inhibitor SL327 than cued fear. Collectively these data support the hypothesis that different levels of the expression of pMAPK/ERK may contribute to

the behavior of high and low fear individuals. These data suggest that different numbers of amygdala neurons undergoing phosphorylation of ERK1/2 kinases contribute to different strengths of conditioned fear. These data begin to provide foundations for the understanding and eventual treatment of pathological fear. This understanding may help identify novel ways to predict individuals at risk for fear-related illness and can potentially lead to targeted treatments for fear-related disorders such as PTSD.

INTRODUCTION

How the brain encodes high levels of fear is not fully understood. This question is important because high levels of fear responding are associated with fear-related pathology (22; 47; 89; 90). Recent experimental data in human subjects suggest that individuals with post-traumatic stress disorder (PTSD) have a higher 'fear load' compared to control subjects (89). Experimental conditions inducing a conditioned fear memory in individuals with a higher fear load leads to higher levels of fear response and reduced extinction of fear (89). The cellular and molecular mechanisms underlying the acquisition and consolidation of an associative fear memory are well-described (31; 45; 73; 83). In contrast, the cellular and molecular mechanisms underlying divergent fear memory formation have seen less exploration. To date, the differences in how individuals consolidate variable fear memory strength is unknown and this knowledge may provide more targeted, effective treatments of fear memory disorders. In order to treat fear-related neurobiological illnesses such as PTSD, it is important to understand the mechanisms occurring in those individuals who exhibit strong fear learning or high 'fear load' (58; 89).

The formation of a long-term fear memory is generally considered to be an adaptive neurobiological process that promotes survival (109). A process of learning involving well-described and shared neural circuitry across a large number of species, the encoding and consolidation of a fear memory requires key cellular signaling cascades in discrete brain regions (71; 85; 113). These include the mitogen-activated protein kinase (MAPK) cascade in the lateral amygdala (LA) (7; 119; 123; 129). In rodent models the phosphorylated (activated) form of MAPK (pMAPK/ERK1/2) is required in the LA for the formation of long-term (but not short-term) associative fear memory (29; 38; 118; 123). High and prolonged levels of fear response characterize aspects of post-traumatic stress disorder (PTSD), and these increased responses may be associated with aspects of underlying differences in the neurobiology of Pavlovian fear (58; 89).

In order to begin to identify some of the differences that underlie very low and very high fear response, we used short-term selection to derive a mouse model selectively bred to show robust differences in both contextual and cued Pavlovian fear memory (29; 94; 101). We refer to these as "Low line" and "High line" mice. In order to determine the role of pMAPK in the High and Low line mice, we pharmacologically inhibited the activation of pMAPK with the drug SL327, a selective MEK inhibitor. In the High and Low line mice we tested whether contextual and auditory cued fear memories are differently affected by the selective MEK inhibitor.

METHODS

High and Low Pavlovian fear mouse lines

Short-term selection for contextual fear was used to create outbred mouse lines with robust differences in fear learning (101). Mice were phenotyped and selected at the

Uniformed Services University from a F_8 generation C57BL/6J x DBA/2J AIL (B6D2 F_8) obtained from the University of Chicago (94; 101). Phenotyping was conducted by selecting the mice that exhibited the highest and lowest contextual freezing one day following Pavlovian fear conditioning (10-30% of populations selected to create new breeding generation). Freezing to the CS was used as an additional measure for selection when necessary. Avoiding brother/sister and first cousin pairings, high and low line breeding pairs were created and represented twelve families per line. Offspring were fear conditioned at 8 weeks of age, selected for high and low contextual fear and high and low line breeding pairs were again formed. This process continued for 3 selection generations (S1-3). Behaviorally naïve, adult male mice from the 4th selected generation (S4) were used for experiments described here. Mice were randomly assigned to one of two major experimental cohorts which ran in parallel: SL327 or Vehicle (Veh).

Husbandry

Mice were housed 2-5 per cage segregated by sex and line (High or Low) in standard shoebox cages in a climate controlled vivarium on a 12hr light/dark cycle with ad libitum food and water. Behavioral experiments were conducted during the light cycle. All experiments adhered to IACUC approved protocols and procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals.*

Pavlovian fear conditioning

Twenty-eight adult male mice (greater than 2 months, less than 20 months) were used for all behavior experiments. Conditioning chambers (Coulbourn Instruments,

Whitehall, PA) measured 7"Wx7"Dx12"H and were inside sound attenuation chambers. Two distinct environments were created with these chambers in order to represent box "A" and box "B". Alterations to the environments involved changes in flooring (grid shock floor for training and context test; wire mesh non-shock floor for cue test), chamber dimensions and appearance, lighting, and scent (70% isopropyl alcohol cleaning solution versus commercial cleanser). Mice were transported to a holding area free of high human traffic and noise and they remained in this area for 30 minutes prior to being placed into the conditioning chambers. Behavior experiments consisted of 3 days of habituation to "A", training day in "A", context test day in "A", and cue test day in "B" (6 consecutive days).

Pavlovian fear conditioning consisted of one paired tone/shock presentation (CS/tone: 75dB, 5000Hz x 30 seconds +US/foot shock: 0.5mA during final second of CS) over 5 minutes with 90-seconds following CS+US pairing prior to being removed from the box ("post-tone/shock"). Animals were allowed 3 minutes in the chamber prior to administration of any stimuli. One day following fear conditioning, animals were returned to the training context "A" for 5 minutes and freezing was scored. The animals were then returned to laboratory animal medicine (LAM) for one day. The following day, (two days following training), in order to isolate associative cued fear, mice were placed into a novel environment "B" and one tone (CS) identical in quality and duration to the training CS (75dB x 30 seconds) was administered. Three minutes elapsed in the novel environment prior to the administration of the CS and total testing time was 5-minutes. Freezing, an active behavior, is the cessation of all movement except for movements associated with respiration (18). Freezing was measured using FreezeFrame™

(Coulbourn Instruments, Whitehall, PA) automatic scoring and verified by the investigator. Adjustments to threshold were made by the investigator who was blind to group assignment. These adjustments insured that automated software accurately detected freezing and threshold setting varied depending on mouse fur color (gray, light brown, or black) and lighting in each chamber.

Scoring of freezing

Freezing was measured across groups as follows: *Training Day*: During the 30-second tone/shock presentation and at 30-second intervals during the 90 seconds following the tone/shock presentation; *Context Test Day*: at one minute intervals (T1-T5) for the 5 minutes of testing, and *Cue Test Day*: during one minute intervals for the first 3 minutes of testing, during the 30-seconds of the CS presentation, and during the 90-seconds following CS presentation (post-tone). All data are means per group.

Pharmacology

SL327 (Sigma, St Lois, MO) was reconstituted in 100% dimethyl sulfoxide (DMSO) so that dosing (100mg/kg) required approximately 40µl total volume. SL327 was received in powder form and was reconstituted with 500µl 100% DMSO for a final concentration of 1mg/20µl. 100% DMSO in equivalent volumes was administered as vehicle. We found it necessary to keep the total volume administered to less than 150µl (otherwise the mice became sick presumably from DMSO). One hour prior to training, mice received an intraperitoneal injection of either SL327 or DMSO and then returned to their home cage until training.

Immunohistochemistry

One hour following Cue Test, mice were anesthetized with ketamine/xylazine (100mg/kg + 10mg/kg) via intraperitoneal injection and transcardially perfused (Gravity Perfusion System for mouse, AutoMate Scientific, Berkeley, CA) with 20ml 0.9% normal saline followed by 40ml 4% paraformaldehyde (FD Neurotechnologies, Columbia, MD). Brains were post-fixed overnight and transferred to 1X phosphate buffered saline (PBS) until processing for immunohistochemistry. Brains were sliced on a vibratome at 40μm and marked in a consistent manner to indicate rostro-caudal sequence. The section selected for quantification of pMAPK expressing neurons was bregma -1.70 as this is the section in which the LA subdivides into LAd, LAvl, and LAvm (46). Only LAd was quantified as this discrete region was found to have different quantities of pMAPK expressing neurons in the High and Low line mice (29). Free-floating sections (5 per well) were placed in 1% BSA blocking solution for one hour prior to incubation in rabbit polyclonal primary antibody to phospho-p44/42 MAPK (1:250 dilution, Cell Signaling Technology, Boston, MA) x24 hours at room temperature. Following 5 washes in PBS, sections were incubated in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200 dilution, Vector Laboratories) for 30-minutes, washed x4, and then placed in avidin-biotin HRP complex (ABC Elite, Vector Laboratories) for 1-hour. Staining of pMAPK-expressing neurons was achieved by exposing sections to SG chromagen/hydrogen peroxide (Vector Laboratories). Sections were mounted in sequence on Superfrost ™ slides, allowed to completely dry, and then dehydrated in increasing percentages of alcohol (50% x1, 70%x1, 95%x1, 100%x2) followed by xylene. Immunohistochemistry for pMAPK in rat and mouse models is an established technique

in our laboratory. Brain regions that serve as positive and negative controls (e.g. central nucleus and optic tract respectively) are verified during each processing of tissue.

RESULTS

We find in a mouse line of high and low Pavlovian conditioned fear that inhibition of the phosphorylation of MAPK with the MEK inhibitor SL327 reduces the strength of fear memory to the conditioned context (context) and conditioned auditory cue (cue). We find that SL327 has a more profound effect on context memory than on cue memory. Additionally, data suggest that High line mice may be more susceptible to SL327 compared to Low line mice. High and Low line mice were randomly divided into either drug (SL327; n=10 High line and n=4 Low line) or vehicle (Veh; n=11 High line and n=3 Low line) group.

Acquisition of conditioned fear - training day

Training Day freezing revealed no differences in animals that received the drug (SL327) or vehicle (Veh) one-hour prior to training (see Figure 3.1). Freezing was measured for the 30.0 sec during tone presentation (which co-terminated with a 1.0 second foot shock) and during 3 x 30.0 sec post shock epochs (total 4 x 30.0sec epochs, 120 sec). A mean freezing score was calculated per group (percent freezing out of the total time measured).

High and Low line animals acquired different levels of Pavlovian fear during training. There was no significant effect of drug during training for either High or Low lines. A 2-way analysis of variance revealed a significant effect of Phenotype $F(1, 23) = 5.409$, $p = 0.0292$. Animals administered vehicle from the High line group showed 16.62 +/- 2.78 freezing compared to animals from the Low line group administered vehicle with

5.0 +/- 2.361. Post hoc analysis of High and Low line animals administered vehicle showed a significant difference on one-tailed t test p = 0.0302. These results suggest that the selected High line exhibits greater freezing during the acquisition of conditioned fear compared to Low line and that SL327 did not have a significant effect during training (Figure 3.1).

Context test

Upon return to the training context one day following training, mice that received SL327 one hour prior to training exhibited less freezing than mice that received vehicle (Figure 3.2). Freezing was measured for each minute during a 5 minute test. A mean freezing score was calculated (percent freezing out of 5 minutes). 2-way analysis of variance identified a significant effect of Drug F (1, 24) = 9.013, p = 0.0062. Post hoc analysis identified that High line animals were significantly reduced: SL327 0.33 +/_ 0.179 versus vehicle 23.12 +/- 5.19, one tailed t test p = 0.0003. Post hoc analysis identified that Low line animals were significantly reduced: SL327 0.7 +/- 0.702 versus vehicle 7.39 +/- 0.663, one tailed t test p = 0.0006. These data show that SL327 significantly reduced freezing in both High and Low lines (Figure 3.2).

Cue test

In order to determine the degree to which the mice learned that the tone predicts a footshock, they were individually placed into a novel conditioning chamber ("context B") and administered one tone identical to that which was administered during training. Freezing was measured for the 30.0 sec during tone presentation and during 3 x 30.0 sec post shock epochs (total 4 x 30.0 epochs, 120 sec). A mean freezing score was calculated (percent freezing out of 120 seconds). 2-way analysis of variance identified a significant

effect of Drug F (1, 24) = 4.494, p = 0.0445. Post hoc analysis identified that High line animals were significantly reduced: SL327 9.25 +/- 3.27 versus vehicle 24.49 +/- 5.83, one tailed t test p = 0.0196. Post hoc analysis identified that Low line animals were not significantly reduced: SL327 3.66 +/- 2.677 versus vehicle 15.509 +/- 6.59, one tailed t test p = NS (0.0609). These data show that SL327 significantly reduced freezing in High line but not Low line animals (Figure 3.3).

Normalized freezing to context versus cue

The data from context and cue memory tests (above) suggest that SL327 had more effect on context memory than on cued fear memory for both the High and Low line animals. We performed a secondary analysis in order to directly compare the effects of SL327 on contextual and cued fear memory. As a measure of change resulting from SL327 we compared context fear to cue fear following drug normalized to its own vehicle control. We identified a significant difference between normalized High line SL327 context test 1.42 +/- 0.81 versus High line SL327 cued test 37.79 +/- 14.1, two tailed t test p = 0.014274. In contrast there was no significant difference between Low line SL327 contexts 23.6 +/- 19.94 versus Low line SL327 cued 10.15 +/- 10.96, two tailed t test p = NS (0.520185). These data indicate a potential difference between High and Low line animals in their sensitivity to SL327, a systemically administered MEK inhibitor (Figure 3.4).

pMAPK expressing neuron number in the LAd following cue test

In the final analysis we measured the effect of SL327 on the number of neurons expressing pMAPK in one specific section of the dorsal sub nucleus of the lateral amygdala (LAd) in High line animals following the recall of conditioned fear.

Immunocytochemistry for pMAPK identified pMAPK expressing neurons in both SL327 and Vehicle administered animals. We found a significant difference in the number of pMAPK expressing neurons between the SL327 animals: 2.500 ± 0.5000 n=2 versus the vehicle animals: 22.00 ± 1.732 n=3, one tailed t test p = 0.0017. These data indicate that SL327 inhibited the activation of MAPK in neurons in a specific section of the LAd (figure 3.5).

DISCUSSION

In these experiments we tested whether there was a different effect of pMAPK inhibition (using the MEK inhibiter SL327) prior to Pavlovian fear conditioning between selectively bred High and Low line mice (29; 94; 101). Second, we tested whether there was a different effect of the pMAPK inhibition in different aspects of Pavlovian fear memory. We used freezing to conditioned contextual and auditory cues as an index of memory acquisition, consolidation and strength (29; 58). We found that the systemically administered MEK inhibitor SL327 did not significantly affect the acquisition of Pavlovian fear during training. In contrast, long term memory for contextual fear conditioning tested 24 hours following training was significantly reduced in both High and Low line mice. For long term cued fear memory tested 48 hours following training, High line mice but not Low line mice showed a significant reduction in fear memory. We further explored this finding by comparing normalized levels of context and cue fear memory following SL327 administration. We found that High line animals showed a significantly different level of freezing between context and cue memory tests, while Low line animals did not. Collectively these data suggest that while context memory is susceptible to MEK inhibition in both High and Low lines, cued fear memory is

susceptible only in High line mice (29). We then asked whether these behavioral differences were associated with a reduced number of pMAPK-expressing neurons in the LAd. We found a significant reduction in the number of pMAPK expressing neurons in the LAd in SL327 treated animals compared to vehicle controls. This finding suggests that the LAd, a key site for cued and contextual fear memory, is directly affected by the systemic MEK inhibitor and that activation of MAPK is inhibited in High line mice.

In the present experiments we confirmed the selected High and Low lines and further identified key aspects of the cellular mechanisms that underlie the phenotypes (29; 58; 94; 101). High line animals show more freezing during training compared to Low line animals and there is no significant effect of drug. These data confirm the selected fear phenotype (29; 58; 94; 101) and suggest that the MEK inhibitor is selective for the consolidation of memory (38; 119; 123). We find that SL327 reduces contextual fear memory in both High and Low lines but cued fear memory is significantly reduced only in High line mice. Importantly we find that SL327 can reduce cue fear memory in High line animals into the range of fear memory of Low line animals (Figure 3.3). These data suggest potential differences in sensitivity to inhibition between both context and cued fear memory and importantly between High and Low lines. A technical limitation of the conclusions may be the imbalance of High and Low line mice available to make up the groups. On the other hand, the robust effect of SL327 on context memory in both phenotypes compared to the reduced effect of SL327 on cued fear memory in the High line animals and no significant effect of SL327 on cued fear in Low line animals may have relevant implications. These findings suggest specificity for both a reduced effect

of SL327 on cued fear across the phenotypes and further suggest that High line animals may be more sensitive to a drug that inhibits plasticity.

These data identify potential differences in sensitivity of cued fear memory compared to context memory following MEK inhibition in a mouse model of High and Low Pavlovian conditioned fear. Additionally, data suggest that higher fear individuals may be more sensitive than lower fear individuals to the effect of MEK inhibition on cued fear memory. A potential explanation for the difference in sensitivity to context versus cue memory may be that during the breeding and selection of High and Low line mice, the primary selection of animals was based on contextual freezing. Thus, we may have 'selected' lines with more robust genetic and cellular differences driving contextual memory. Alternatively, it may be that the hippocampus is more sensitive to the effects of SL327 as it is a much larger structure than the lateral amygdala. In order to test the involvement of the lateral amygdala in the behavioral effects observed, we measured the number of pMAPK expressing neurons in the dorso-lateral amygdala (LAd). These anatomical data support the hypothesis that high fear is mediated, at least in part, by more pMAPK in the LAd and that this increase is more sensitive to MEK inhibition. Collectively these data suggest that higher fear individuals may be more sensitive to intervention or treatment with MEK inhibitors compared to lower fear individuals, particularly in contextual fear memory.

Pavlovian fear conditioning has been proposed to underlie key aspects of memory formation associated with anxiety disorders including PTSD (58; 138). Thus, understanding the cellular mechanisms underlying Pavlovian fear conditioning has the potential to lead to the development of targeted behavioral and pharmacological

mechanisms aimed at inhibiting the consolidation and reconsolidation of fear memories (38; 39; 58). The MAPK cascade has been identified as an essential molecular pathway in Pavlovian fear consolidation in rats (12-14; 119), and mice (29; 38; 123) and also in reconsolidation of fear memories (14; 53). Recent experiments in humans with PTSD have identified increased strength of consolidated fear memory and a decreased rate of extinction (118). This increased Pavlovian fear memory strength, termed 'Fear Load' (118), suggests that individuals with PTSD may have different genetic and cellular mechanisms driving fear memory strength.

In these experiments we investigated an aspect of the 'fear load' hypothesis by testing whether animals known to develop different levels of Pavlovian fear memory are differently susceptible to inhibitors of MAPK memory consolidation cascade. The present data show that cued fear memory in High line animals is sensitive to inhibition by MEK inhibitors. This finding suggests that an increase in the number of pMAPK neurons or quantity of pMAPK is associated with the high fear phenotype. This conclusion is supported by our previous data showing that High line mice have a higher number of pMAPK expressing neurons in the LAd following Pavlovian fear conditioning (41). Collectively these data point to a hypothesis of an increased network of activated neurons in the LAd in High line mice and higher fear load individuals (56; 58). This increased network of neurons may suggest that an element of fear memory strength is governed by both the number and specificity of neurons encoding the fear memory in the LA (56; 58).

This study identified potential differences in distinct types of memory tests that may be more or less sensitive to pharmacologic inhibition. If a MEK inhibitor was used in a

clinical setting for trauma associated memories, these data suggest that memories of the trauma context may be inhibited more than memories of trauma-associated cues (i.e. sights, sounds, smells). More studies in animal models are needed to further test and confirm these findings. These data suggest that SL327 can be used in a mouse model of High and Low Pavlovian conditioned fear to reduce high fear memory to the level of low fear memory. However, they also show that contextual and cued fear memory may be differently susceptible to inhibition by MEK inhibitors. Thus the usefulness of MEK inhibitors for clinical use may depend on the traumatic fear memory being treated. Further work is needed to extend these finding and to explore the new hypothesis raised by these data.

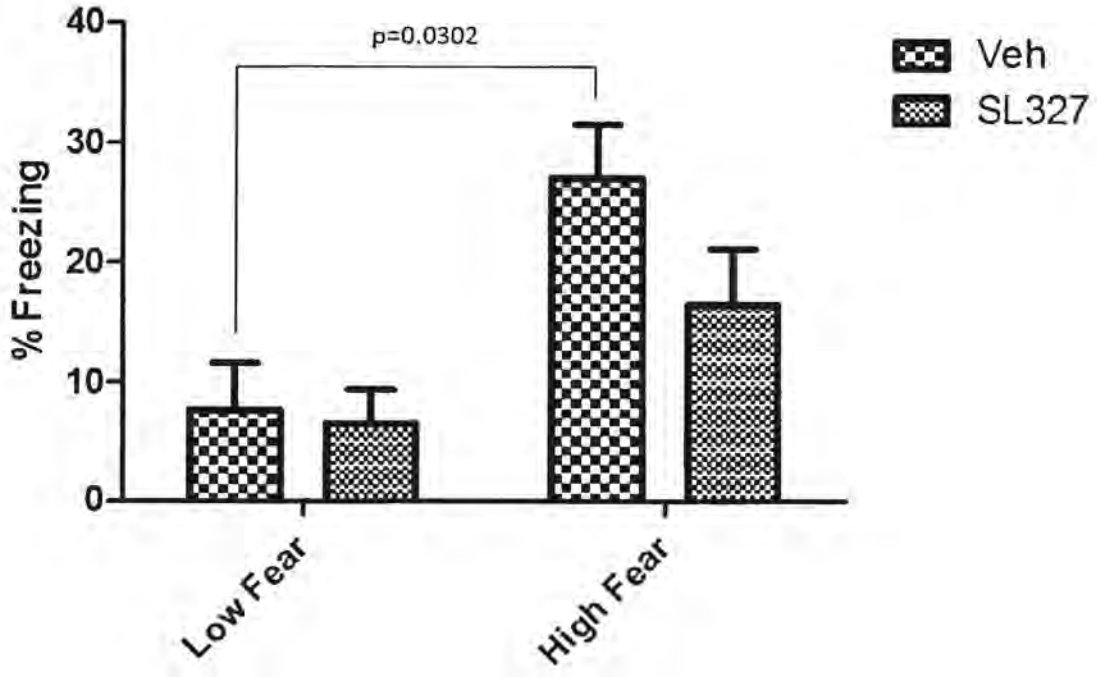


Figure 3.1. High and Low fear mice show different levels of fear memory immediately following training. High fear vehicle control mice show more fear than Low fear vehicle control mice. There was no significant effect of the MEK inhibitor SL327 during training. 2-way ANOVA reveals an effect of phenotype (p=0.0302).

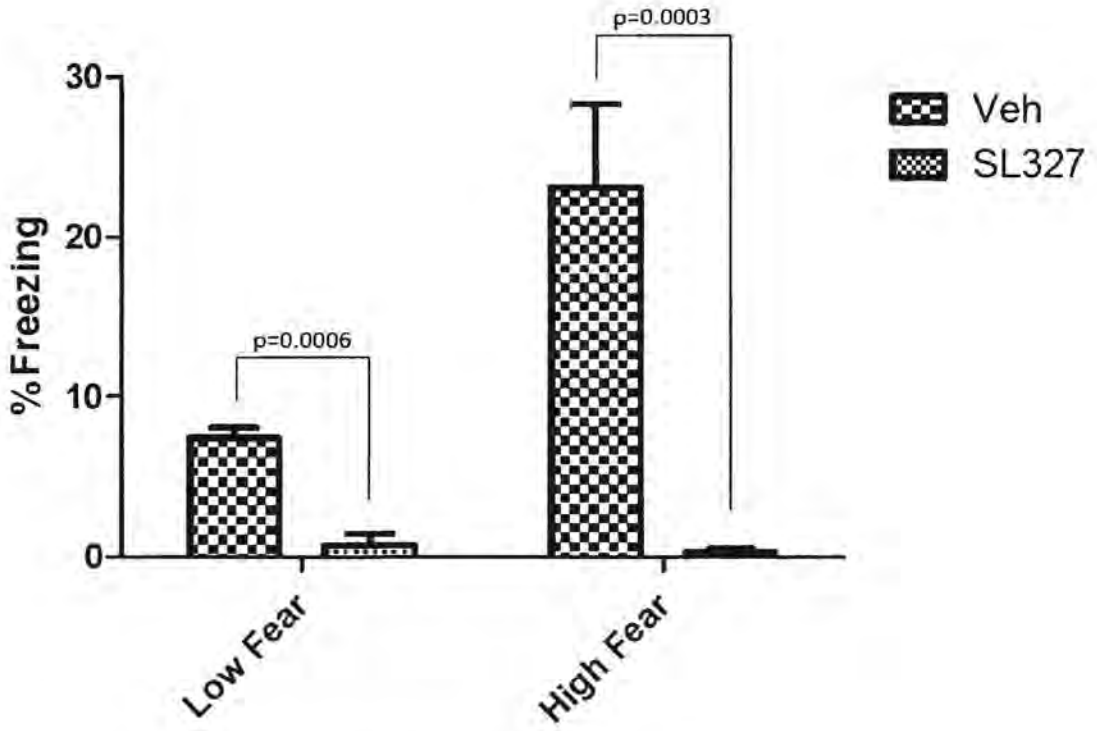


Figure 3.2. The MEK inhibitor SL327 abolished contextual fear in both High Fear and Low Fear mice. SL327 was administered prior to initial fear conditioning. Contextual fear memory tested 24-hrs following initial fear conditioning consisting of 5 minutes in the training context. 2-way ANOVA reveals an effect of drug ($p=0.0062$).

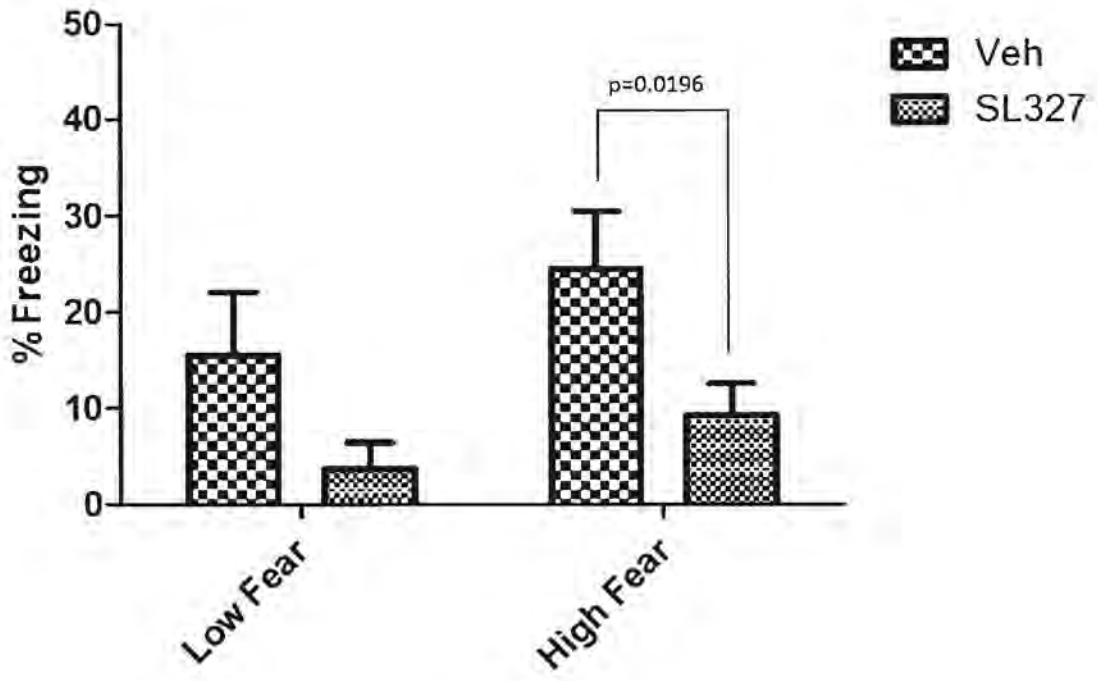


Figure 3.3. The MEK inhibitor SL327 inhibited cued fear conditioning in high fear mice. There was also a non-significant reduction in fear in Low Fear mice. Levels of fear to the conditioned cued were tested 48-hrs following initial fear conditioning. SL327 was administered prior to conditioning. 2-way ANOVA revealed an effect of drug ($p=0.0445$).

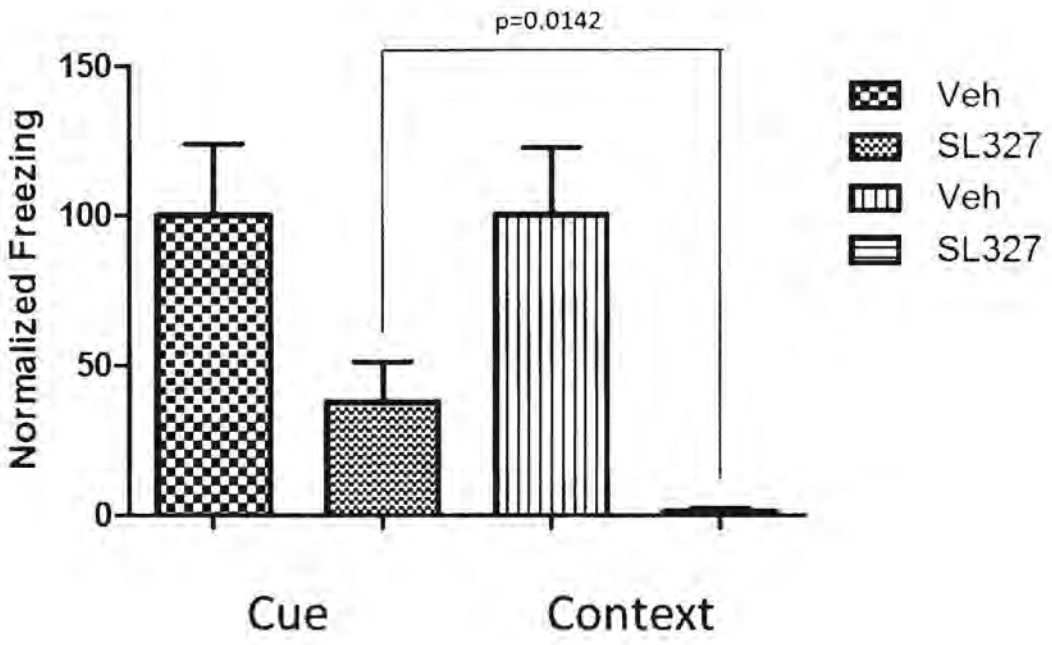


Figure 3.4. Comparison of the level of cued and context fear in High fear mice following SL327. Freezing is normalized to vehicle control. Post drug normalized Cued and Context fear measures are significantly different. Contextual fear is completely abolished by SL327 however cued fear was only reduced to ~ 50% of its control.

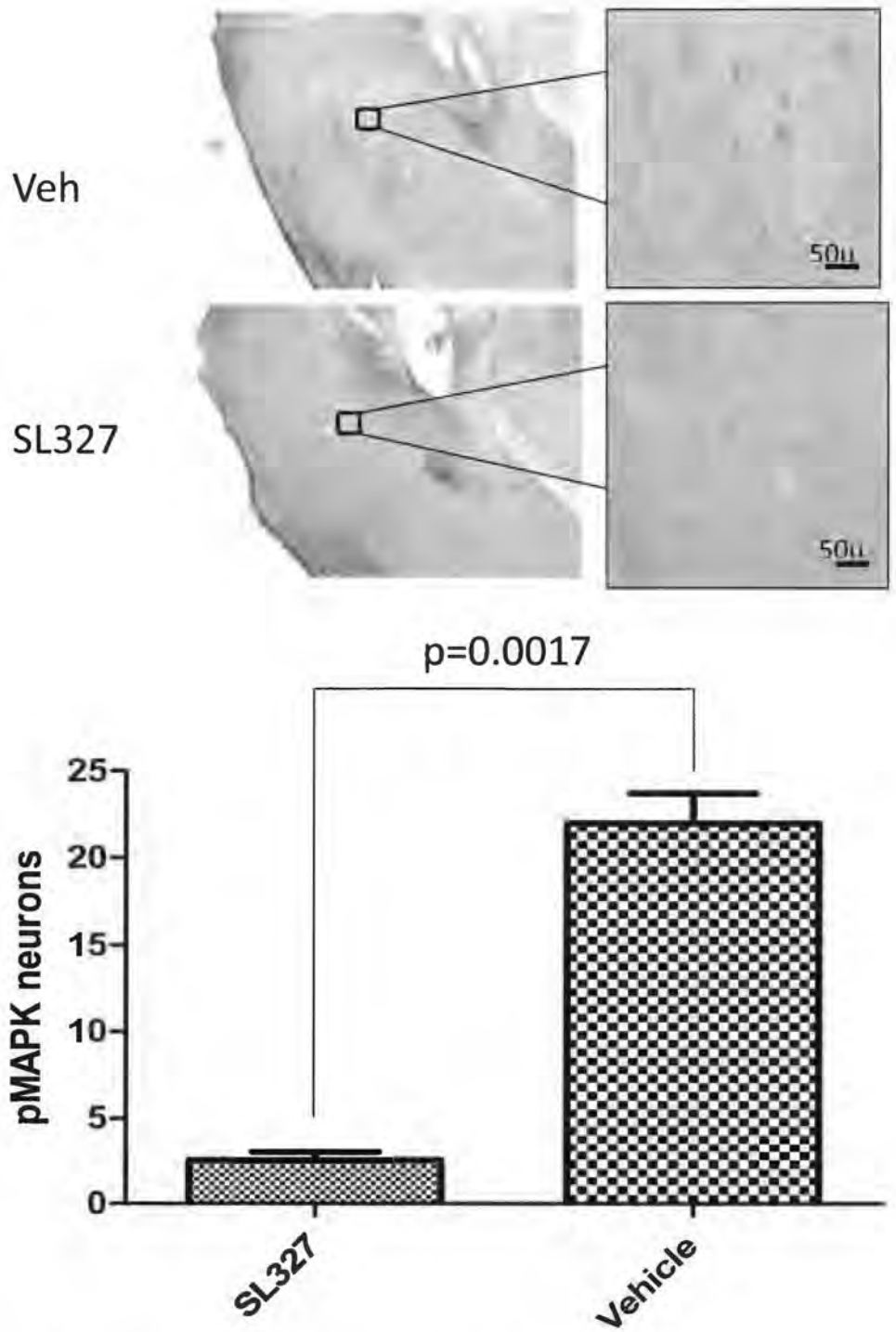


Figure 3.5. pMAPK-expressing neurons in the LAd one-hour following cue fear test (reconsolidation) in mice that received SL327 (n=3) vs. mice that received vehicle (n=3) prior to fear conditioning. The systemic MEK inhibitor SL327 reduces the number of neurons expressing pMAPK in the lateral amygdala. In High fear animals, 60 min following cued memory test, the number of neurons in the dorsal sub-nucleus of the lateral amygdala (LAd) expressing pMAPK, was significantly reduced.

CHAPTER 4: Discussion

SUMMARY OF FINDINGS

Hypotheses

A widely accepted requirement for the consolidation of an associative fear memory is the expression of phosphorylated mitogen activated protein kinase (pMAPK) in the lateral nucleus of the amygdala (119; 120). Based on this, we asked whether mice selectively bred to exhibit high Pavlovian conditioned fear show greater expression of pMAPK in the LA following a single fear conditioning trial. We hypothesized that compared to Low line mice and all controls, Pavlovian fear conditioned High line mice would have the greatest expression of pMAPK in the LA. Further, we hypothesized that inhibition of pMAPK prior to fear conditioning would effectively block the consolidation of fear memory, and that High line mice would exhibit fear memory behavior similar to Low line mice. We therefore hypothesized that fear-induced plasticity in a key locus of fear memory acquisition and storage underlies divergent fear phenotypes.

Main Findings

Our findings support our hypotheses. We found that High line mice have more pMAPK-expressing neurons in the dorsolateral amygdala (LAd), a discrete subdivision of the LA not at baseline but following fear conditioning. This finding suggests that precise network differences exist between the two fear phenotypes (41). Further, when we administered a MEK inhibitor that interrupts the MAPK signaling cascade and blocks the phosphorylation of MAPK, High line mice exhibited fear memory strength similar to

that of Low line mice. In these experiments we show that one mechanism of high Pavlovian fear memory is increased activity-dependent plasticity in a key locus of fear memory acquisition and consolidation. This initial study of phenotype and fear memory identified potential differences in two types of memory tests (context and cue). Results suggest that contextual fear memory may be more sensitive to pharmacologic inhibition compared to cued fear memory. SL327, a selective MEK inhibitor, reduced freezing in the High line mice to that of the Low line mice. Interestingly, a differential reduction was seen between contextual and cued fear memory as a result of administration of SL327. An unanswered question is whether the hippocampus is more susceptible to the effects of the systemically-administered drug. We quantified pMAPK-expressing neurons in the LAd one hour following cue fear memory test. Results show a significant reduction in the quantity of pMAPK-expressing neurons in the LAd in the SL327 group compared to the vehicle group. Quantification of pMAPK neurons in the hippocampus is suggested for future studies. More studies in animal models of high and low fear are necessary to further test and confirm these findings. An additional finding is that High line mice have a unique pattern of pMAPK-expressing neurons within the LAd compared to Low line mice and that this may also underlie the high fear memory behavior (see appendix A). The specific 'map' of neurons expressing pMAPK may reveal key underpinnings of what leads to divergent fear memory and consequently, fear response (13). These data can aid in our identification of distinct subpopulations of neurons within the LAd that are involved in determining the strength of an acquired fear memory. A currently unpublished experiment revealed an additional interesting finding in the High and Low line mice. We maintained a small cohort of mice in our LAM that had

undergone Pavlovian fear conditioning and the subsequent two days of testing. We were interested in seeing whether the High line mice continued to exhibit higher freezing compared to Low line mice upon reconsolidation of an associative fear memory. Thirty days following training, mice were placed into Context "B" and one tone precisely like the tone that was administered during training was administered (30 seconds, 75dB, 5000 Hz). Analysis of freezing data revealed a significant difference in cued fear between the High and Low lines one-month after the initial fear learning experience (see Appendix B). This finding is one that should be further explored in future experiments. These data could contribute to the discovery of mechanisms directly involved in the storage of persistent fear memory that does not extinguish over long periods of time, a hallmark of PTSD. Understanding fear processing and the mechanisms that underlie persistent high fear memory at the microcircuit level can vastly improve available treatment options for fear-related illness. Further suggested follow-on studies will be discussed in a later section.

THE MICRO CIRCUITRY OF FEAR

The Amygdala and the Relevance of LAd

Fear responses prime the body to flee or fight danger and are therefore critical to survival. The experience of fear requires an intact amygdala with intact sensory inputs (31; 71; 134). Humans are able to verbalize their experience of fear. In contrast, in non-human animals, investigators rely upon observation of well-characterized defense responses and physiologic alterations to suggest that the animal is 'experiencing' fear (73). However, we are unable to determine with certainty that non-human animals experience the *emotion* of fear rather than simply respond to the real or perceived threat

(73). Nonetheless, fear behavior and response is seen in numerous vertebrate and invertebrate species and has been studied in organisms as simple as the fruit fly and as complex as humans (19; 73). Lesion studies in laboratory animals as well as case reports of human amygdala damage have demonstrated the importance of the amygdala in fear processing and storage (1; 4; 31; 67). The lateral amygdala (LA), one of approximately a dozen subnuclei that make up the amygdala, has long been established to be the site of sensory information convergence following an emotionally salient experience (76; 112; 131). These sensory signals arise from the sensory thalamic and cortical brain regions as part of a highly conserved fear circuitry that serves to rapidly alert an organism to threat (2; 73; 112). Remembering our most fearful or life-threatening experiences allows us to adapt, predict future consequences, and ultimately these memories serve to promote survival (33; 109). In order for our memories (to include fear memories) to be stored long-term, changes in the form of plasticity are required in key locations of the central nervous system (53; 126). One form of this plasticity results from the phosphorylation (activation) of MAPK in the LA (119). Three subdivisions of the LA include the LAd, LAvl, and the LAvm. Important circuitry within these subdivisions has been unraveled and we now understand signaling from the LAd to LAvl and LAvm to be unidirectional whereby LAd signals to LAvl and LAvm without reciprocation and no signaling between LAvl and LAvm has been found to exist (99). Thus, the LAd has been suggested to be the key division involved in the initiation of fear-induced plasticity (99). Based on this knowledge, we asked whether mice that exhibit high Pavlovian conditioned fear have greater expression of pMAPK in the lateral amygdala at baseline and/or following fear conditioning. Interestingly, it is this key region within the LA that we find evidence of

differential plasticity between the High and Low line mice following Pavlovian fear conditioning (29). These data provide important insights into the micro network mechanisms encoding both normal and potentially pathological fear and begin to explain differences in fear processing at the point of fear acquisition. Understanding where divergent fear memory strength begins within the neural circuitry will aid in our ability to effectively treat fear-related illness.

Intra-LA circuitry

Signaling through the LA is largely unidirectional (99). LAd shows connectivity to LAvl and LAvm but LAvl and LAvm do not signal back to LAd. Further, LAvl and LAvm do not show connectivity to each other. Projections from other subnuclei of the amygdala into the LA are largely reciprocal and are initiated in the medial division (LAvm). The LA therefore is primarily a site of signal input and vast signal output (99). Within the LA, the LAd in particular receives information about an emotionally-salient event and channels those signals throughout the amygdala and to cortical and subcortical brain regions where processing and response are initiated (99). Our finding that differential fear-induced plasticity between High and Low line mice is isolated within the LA to the discrete LAd subdivision is in-line with this site as a key initiator of plasticity. Mice that exhibit high fear memory have increased plasticity in the LAd and no other LA subdivision. It is important to also quantify pMAPK-expressing neurons in other amygdala subnuclei such as BA and Ce to determine whether increased plasticity is the overall finding within the amygdala in general. This would provide a clearer picture of the mechanisms that underlie individual high associative fear learning.

Hippocampal to LA connections

The hippocampus is a brain structure that plays a crucial role in memory (110; 130). Best illustrated by cases in which the hippocampus is damaged or lesioned, we understand this temporal lobe structure to be crucial for what are known as declarative memories such as recalling facts and past events (107; 110). In human and non-human animals, the hippocampus allows for the memory of spatial/contextual information, key for survival particularly in the wild (110). Contextual fear memory is important as it allows us to predict threat in given environments based on previous experience. During fear conditioning, information about the context synapses in the hippocampus where that information begins processing for short and/or long-term storage (consolidation) (Swanson and Petrovich, 1998). From the hippocampus, contextual information is relayed to the LA and BA where signals converge with sensory input about the aversive nature (e.g. foot shock) of the experience (82). Therefore, emotional significance is given to the context as a result of the convergence of signals (in the LA) between the context and the aversive stimulus (82). Once consolidation of this contextual memory has formed, the hippocampus is no longer necessary for a fear response (5; 65). If the hippocampus is lesioned prior to Pavlovian fear conditioning, no contextual fear memory will develop (98). That is, when a rodent is fear conditioned in a specific conditioning chamber, no evidence of fear to that chamber exists when returned the following day. Additionally, if the hippocampus is lesioned one day following fear conditioning, fear to the context is no longer evident (5; 65; 81) suggesting that consolidation of contextual memory requires more than a day to occur. The hippocampus plays a critical role in aspects of fear memory, namely contextual fear memory. Recent (unpublished) findings from our lab examined hippocampal volume through the use of MRI and manganese

uptake studies (Bergstrom et al). Data suggest that no volumetric differences in hippocampus exist between the High and Low line mice, however further investigation into hippocampal differences between the High and Low line mice is encouraged. Because our selection for high and low fear was based on primarily contextual freezing, an important question is whether differential plasticity in the hippocampus underlies the divergent fear memory between the two phenotypes.

ADVANTAGES AND DISADVANTAGES OF HIGH AND LOW FEAR MICE LINE

Mouse model of High and Low fear

Understanding the neurobiological mechanisms that underlie different levels of fear response is an important aim in the prevention and treatment of fear-related disorders such as post-traumatic stress disorder. An important and yet unanswered question in the field of fear memory research asks what are the cellular and molecular mechanisms that underlie high and low Pavlovian conditioned fear memory? In order to begin to answer this question, we used a mouse model that exhibits divergent Pavlovian fear memory. This model of high and low fear is advantageous in that it allows the researcher to isolate a specific phenotype of interest and then investigate the mechanisms that underlie the neurobiology of that phenotype (101).

The utility of such a model has been shown in other works when used to isolate specific phenotypes such as anxiety in order to determine underlying mechanisms responsible for that phenotype (50). The use of knockout or selectively bred animal models allows the researcher to target a specific condition or behavior of interest and interrogate the underlying cellular, molecular, and genetic underpinnings of that specific interest (27). The cause of PTSD in humans is multifactorial (11; 49; 132). However,

gaining a solid understanding of the conditions under which an individual is more susceptible to developing PTSD can greatly impact our ability to predict, capture, and treat these illnesses. Recent research suggests that individuals that exhibit a higher 'fear load' may be more susceptible to developing fear-related illness such as PTSD (89). Thus, an animal model of high fear load will allow for the study of neurobiological differences that underlie this phenotype and advance our clinical capabilities. In this work we used short-term selection for contextual fear to create an outbred mouse line of high and low Pavlovian fear memory (101). In order to phenotype this line, we selected the mice that exhibited the highest and lowest contextual freezing one day following Pavlovian fear conditioning (10-30% of each generation). Selected animal lines developed from an advanced intercrossed line (AIL) allow for increased specificity in isolating genetically determined behavioral and physical phenotypes due to a breakdown in linkage disequilibrium (58; 94; 101). Traits that are not associated with the behavior of interest remain equally distributed between the lines while traits that drive the phenotype will segregate with the behavior (94; 101).

Over a century of interest and inquiry has led to our current understanding of fear memory, a process of learning that requires plasticity within specific networks within the brain and new protein synthesis. An explosion of research began on the neural correlates of fear memory processing in the late 1980's and these works allow us to now ask more specific questions such as the one posed in this research. Understanding how individual levels of fear memory strength develop within the known mammalian fear circuitry is an important aim in science and psychology.

Inter-trial Interval Freezing

An interesting finding that deserves exploration is the inter-trial interval freezing. Freezing during the period of time following a tone/shock (or during the tone alone in cue test) but before the next tone/shock revealed interesting data (unpublished data, Coyner et al 2012). High line mice exhibit high freezing during the fear-inducing cue and they continue to exhibit very high freezing after the cue is no longer present. This sustained level of high freezing may describe the high fear phenotype, however, the neural correlates of inter-trial interval freezing are not known. An interesting experiment would be to vary the length of inter-trial intervals and look for differences in freezing during those specific times between the two lines. Fear to specific cues that predict threat is evolutionarily adaptive. However, when the cue is no longer present, it is not advantageous to continue to respond in a defensive manner. High freezing during this period of time may model aspects of fear pathology and should be the focus of future research.

Future Research with the High and Low Line Mice

In order to begin to unravel the neurobiological mechanisms that underlie high individual fear behavior following a fearful and/or traumatic experience, we examined a marker of plasticity in the LA of mice that exhibit high Pavlovian fear memory. Our findings suggest that in High line mice, increased plasticity in the LAd, a key locus of sensory signal convergence and a highly discrete region of the amygdala, follows Pavlovian fear learning (29). This is an important first finding; however, more must be investigated in order to fully understand the divergent fear phenotypes. An important question is whether non-associative fear memory yields the same differential plasticity in

the LA. This can be examined by adding a "shock alone" group in which no auditory tone predicts the footshock. Previous research in rat (13) suggests that this control group does not generate the same degree of plasticity (as detected by pMAPK expressing neurons) as the tone/shock group. However, mice exhibiting High and Low fear memory may reveal different results.

Another question we would ask is whether pMAPK is the ideal marker of plasticity to examine in the LA. By quantifying pMAPK-expressing neurons, we are unable to determine whether there is an important upregulation of a protein upstream or downstream of that marker. Neuronal signaling cascades involve a plethora of receptors, second messengers, protein kinases, transcription factors, and intermediate early genes (3). There is value in looking at other proteins upstream of pMAPK that may be driving the MEK pathway. Likewise, markers of plasticity downstream of pMAPK that generate new protein synthesis are worthy of investigation.

When a threat is no longer present, the ability to no longer respond to sensory cues that previously predicted that threat is an important component of healthy fear response and recovery (125). This concept is known as fear extinction and represents 'new learning' (87). Individuals that develop PTSD are thought to have deficits in this process (22; 47). In fact, some researchers and clinicians suggest that PTSD is a disruption in the ability to forget rather than a disease of remembering (132). Indeed, there are neurobiological reasons why remembering the cues that predict threat is important. However, over time, when those memories no longer serve us well, there are neurobiological benefits to ceasing to maintain a state of hypervigilance (88). Individuals who suffer from fear-related illness also suffer multiple co-morbidities such as

depression, suicidal ideation, and drug abuse (22; 63). Managing our fear memories so that they alert us to threat and yet, stay in context is ideal. For these reasons, an important experiment to design and carry out on these High and Low line mice is one that examines differential extinction and its underlying neural correlates. Extinction of fear memory is known to involve connectivity between the amygdala and prefrontal cortex (87). Differential signaling between these two brain structures in the High and Low line mice may underlie important aspects of their divergent fear behavior.

Lastly, investigation into how long the High line mice exhibit higher freezing levels compared to Low line mice is an important question related to PTSD symptomatology. PTSD is characterized by high and prolonged levels of fear response (89; 90). In order to arrive at a true model of PTSD, it is important to ensure that prolonged differences in behavioral measures of Pavlovian fear exist.

Benefit to PTSD Research

Fear is an individual experience and how strongly an individual processes and stores a fear memory determines subsequent responses to the cues that trigger that memory (66). Learning is the fundamental process that leads to memory including fear memory (21). The fundamental neural circuitry of fear memory is well understood, as are some of the underlying cellular and molecular mechanisms that underlie fear memory acquisition and consolidation (2; 74; 93; 103). However, an understudied and poorly understood question is how high and low fear is acquired and consolidated. In humans and rodents (to a lesser degree), a broad range of fear responses to a given experience exists. How one responds to a fear-evoking experience is important in whether that fear

is beneficial or pathologic. The majority of individuals that experience extreme fear and/or trauma do not develop post-traumatic stress disorder (64). Conversely, most individuals that experience intense fear with or without trauma experience a non-pathologic response known as 'acute stress disorder' (ASD) (91). This normal variant allows an individual to process the experience and relegate it to an appropriate place and in a short period of time (less than thirty days) fully recover from the psychological impact. This differential fear response is an important area of investigation
Treatment options for PTSD currently focus on cognitive behavioral therapy (CBT) and pharmacologic interventions (124). CBT uses the tool (concept) of extinction whereby repeated exposure to specific fear-related triggers or cues are no longer associated with the fear-evoking stimulus leading to 'new learning' that breaks the associative memory of CS/US. Pharmacologic interventions which are variably effective have a variety of mechanisms for reducing the impact of PTSD but have not been overwhelmingly effective (30; 42; 124). The ability to understand the processes that occur at the time of the acquisition and early consolidation phase that lead to a particularly strong fear memory is required in order for providers to more accurately target the precise mechanism that underlies fear memory that results in pathology.

Future research on PTSD

Pavlovian fear conditioning is a form of classical conditioning in which a previously neutral stimulus comes to predict an aversive stimulus (106). This form of associative learning elicits long-term fear memory and models a key aspect of PTSD. PTSD requires the experience of intense fear and actual or potential threat to life (6). Symptoms that lead to the diagnosis of PTSD are re-experiencing, hypervigilance, and

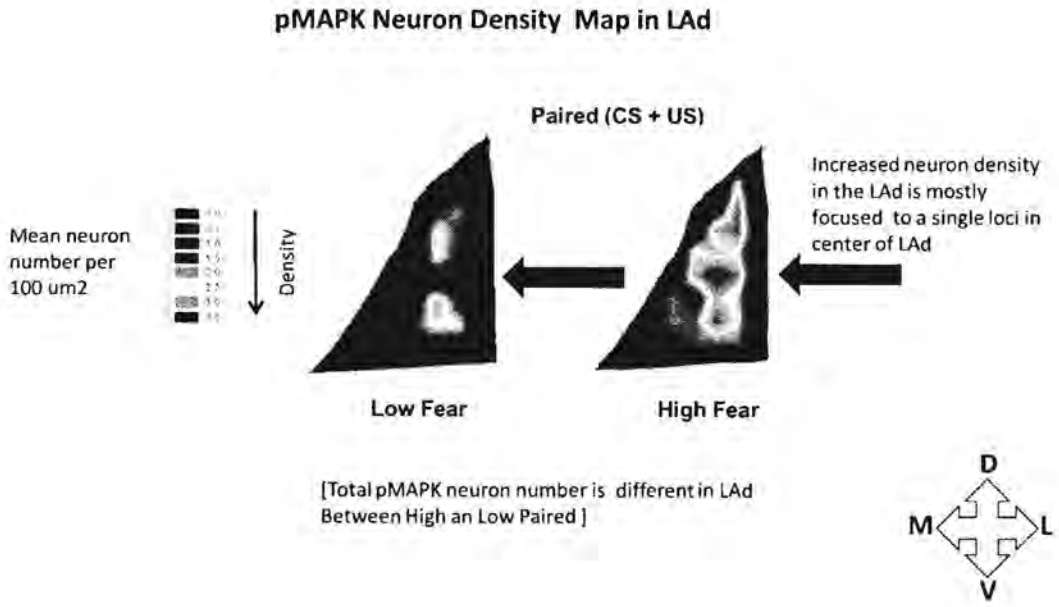
avoidance of reminders of the event. Further, evidence suggests that PTSD may develop as a result of the inability to extinguish the fear memory which occurs when stimuli associated with the trauma no longer predict the trauma (50). Extinction is a form of 'new learning' and undergoes similar cellular and molecular processes that occur during initial consolidation of a fear memory. PTSD has been proposed to be a disease of learning and memory in which a given fear-inducing experience is learned too well and unable to be forgotten (41). Therefore, understanding the cellular and molecular mechanisms as well as the circuit-related dysfunction that occurs in a high-fear-learning/poor extinction model may lead to breakthroughs in PTSD treatment. Here we have discovered that increased levels of a marker of fear learning induced plasticity (pMAPK) in a specific subdivision of the LA is present in mice that exhibit high Pavlovian fear learning as compared to mice that exhibit low Pavlovian fear learning (29). This is an important initial finding and begins to establish how divergent fear behavior develops at the cellular level. However, this is just the beginning and research must continue in order to determine the full nature of the difference in how high fear is consolidated. Further, this work does not examine fear extinction in High and Low line mice. Fear memory circuitry involves the amygdala, hippocampus, and medial pre-frontal cortex and all of these brain regions show dense networking in both human and non-human animals (73; 97; 107). The LA, an undisputed locus of fear memory acquisition and consolidation is the focus of this initial work. High line mice show differences in pMAPK expression in this critical region as compared to Low line mice. As previously stated, the hippocampus serves to establish the contextual significance of a fear-inducing or traumatic experience. Contextual cues about a traumatic experience are

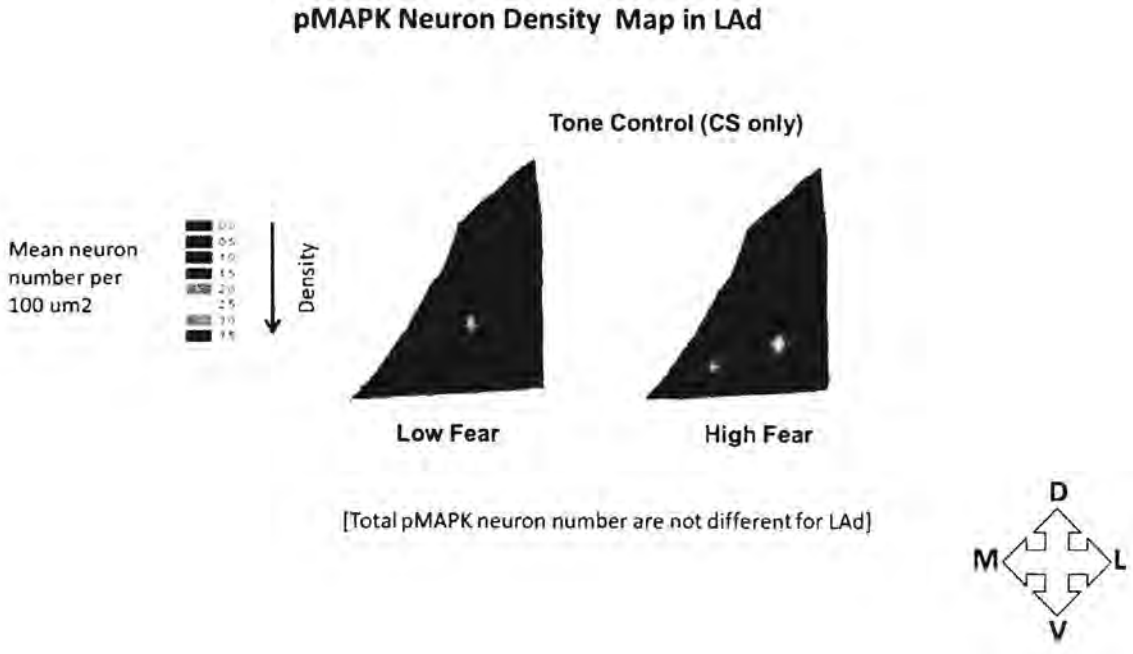
an important component of PTSD as evidenced by the avoidance of places where the trauma took place (11). Future work should explore potential differences in plasticity in this important brain region. Our mice were primarily selected on high or low contextual fear memory (58; 101). For this reason, the hippocampus may show further important plasticity-related differences and should be the focus of future inquiries related to this question. An important question is whether High line mice that receive shock alone (no auditory cue) show the same degree of pMAPK expression in the LAd compared to Low line mice. Previous findings from our lab in a rat model suggest that shock alone groups exhibit less pMAPK in the LAd (12), however, findings may be different in a model of High Pavlovian fear. Another important question to examine in these High and Low line mice is whether behavioral and histochemical differences seen between the two lines continue to exist over time. A hallmark of PTSD is that acute stress symptoms described above do not abate over time, but rather linger for months, years, or for a lifetime (6; 24). Therefore, examination of fear memory beyond one and two days post-training may reveal more information about divergent fear. We examined this question in a very small cohort of mice and data suggest that 30-days following Pavlovian fear conditioning, High line mice exhibit significantly more freezing to cue compared to Low line mice (see figure in appendix B). This is an important finding related to PTSD and should be explored further.

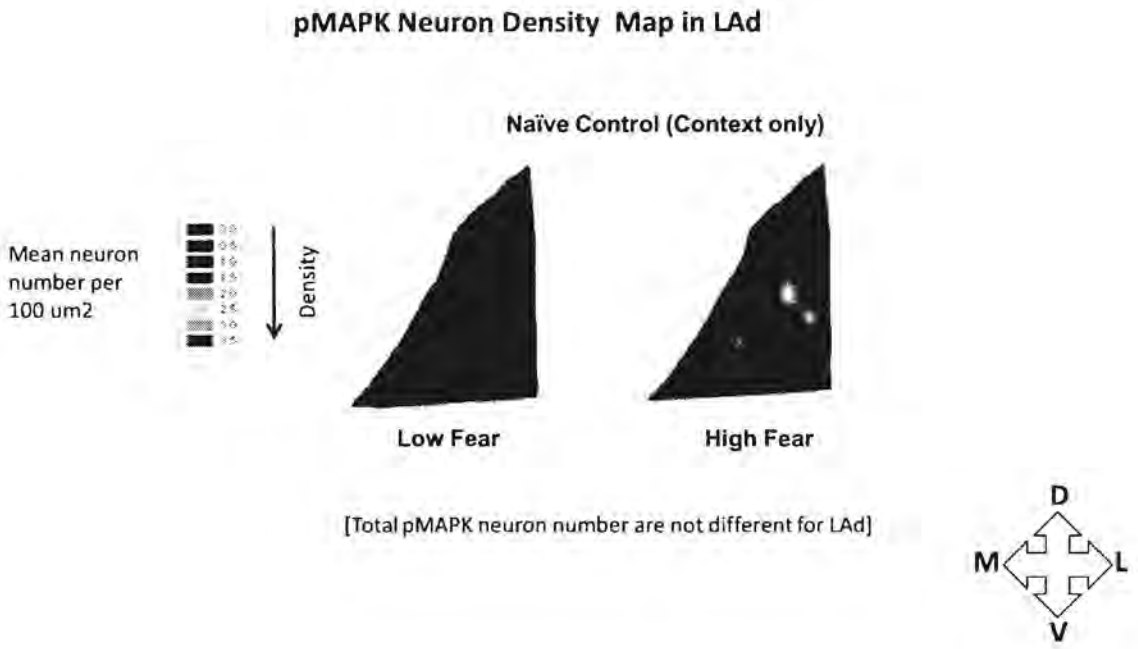
In closing, this work adds knowledge to the field of fear memory research and begins to examine an important question: how do high fear individuals process and store an associative fear memory? In seeking to more effectively treat fear-related illness such

as PTSD, it is important that we understand the neurobiological mechanisms that underlie divergent levels of fear. We hope that future research will expand upon these findings.

APPENDIX A: Density Plots of High and Low Line pMAPK-expressing Neurons

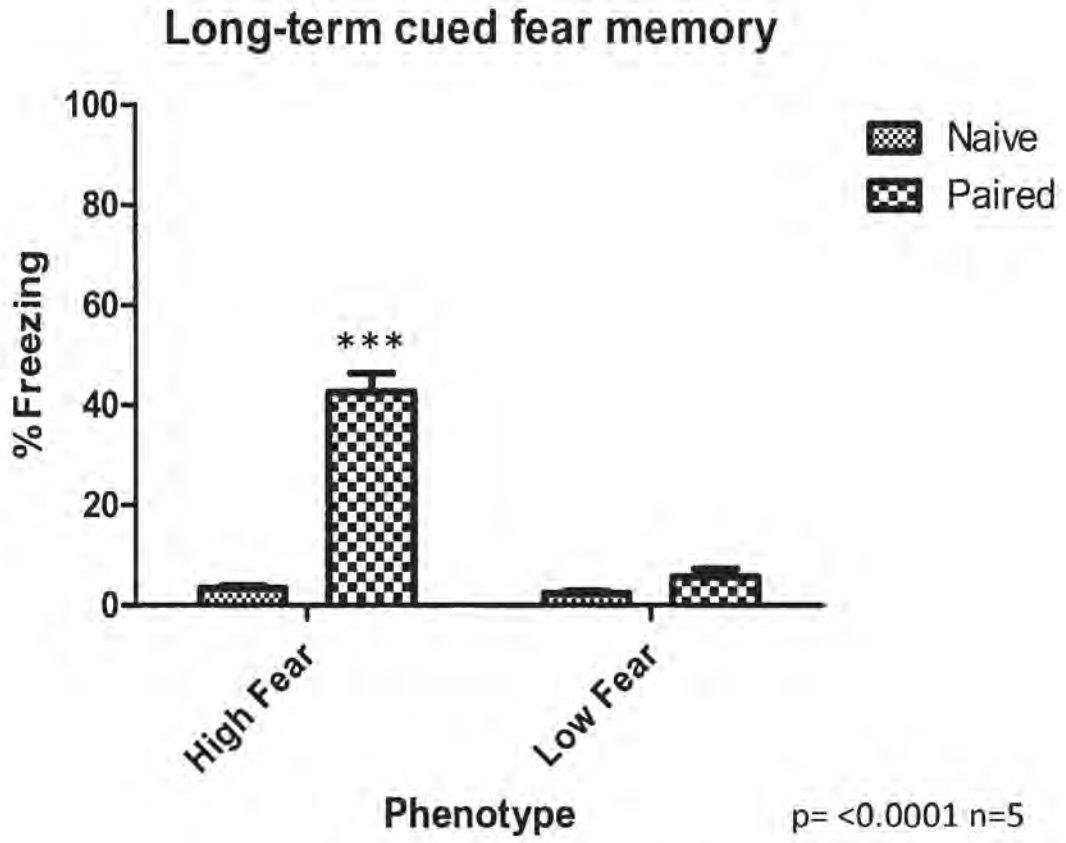






Appendix A: Neuron density plots (heat maps) show the mean distribution of pMAPK-expressing neurons in the LAd in experimental and control groups of High and Low line mice (Paired, Tone Control, and Naïve). A unique topography of pMAPK neuron expression is observed in the High line paired group compared to the Low line paired group with an increased concentration of pMAPK neurons in the mid LAd in the High line that is absent in the Low line (arrow). No difference is seen in overall quantity of pMAPK-expressing neurons in the two control groups. This unique topography of plasticity seen following associative fear learning may contribute to the behaviorally divergent fear memory and help to explain the neurobiology of high fear.

APPENDIX B: Freezing data on long-term fear memory in High and Low line mice



Appendix B: 30-days following Pavlovian fear conditioning, High and Low line mice were returned to "context B" and administered cued fear was examined. High line mice exhibit significantly higher freezing to cue compared to Low line mice who exhibited minimal freezing. these data suggest that over time, Low line mice extinguish the cued fear memory while High line mice maintain a strong cued fear memory.

APPENDIX C: co-authored Published book chapter in "trp channels in drug discovery" vol. 2, chapter 11

Rodent models of conditioned fear: Behavioral measures of fear and memory

Running Head: Conditioned Fear

Jennifer L. McGuire 1,3, Jennifer L. Coyner,1, Luke R. Johnson 1,2,3

1Psychiatry and Neuroscience, School of Medicine, Uniformed Services University (USU), Bethesda, MD, USA.

2Center for the Study of Traumatic Stress (CSTS), MD, USA.

3Center for Neuroscience and Regenerative Medicine (CNRM), MD, USA.

i. Summary/Abstract

Pavlovian fear conditioning is a robust technique for examining behavioral and cellular components of fear learning and memory. In fear conditioning, the subject learns to associate a previously neutral stimulus with an inherently noxious co-stimulus. The learned association is reflected in the animals' behavior upon subsequent re-exposure to the previously neutral stimulus or the training environment. Using fear conditioning, investigators can obtain a large amount of data that describes multiple aspects of learning and memory. In a single test, researchers can evaluate functional integrity in fear circuitry, which is both well characterized and highly conserved across species. Additionally, the availability of sensitive and reliable automated scoring software makes fear conditioning amenable to high throughput experimentation in the rodent model; thus this model of learning and memory is particularly useful for pharmacological and toxicological screening. Due to the conserved nature of fear circuitry across species, data from Pavlovian fear conditioning is highly translatable to human models. We describe equipment and techniques needed to perform and analyze conditioned fear data. We provide two example fear conditioning experiments, one in rats and one in mice, and the types of data that can be collected in a single experiment.

ii. Key Words

Amygdala, associative learning, memory, stress, fear, Pavlov, autoscoring, freezing, conditioned stimulus, unconditioned stimulus, conditioned response, high throughput.

1. Introduction

Classical fear conditioning, also known as Pavlovian fear conditioning, is a robust procedure for evaluating associative learning and memory. As such it is a key behavioral model for testing pharmacologic agents regulating learning and memory. During fear conditioning, the subject learns to associate a previously nonthreatening stimulus (designated the conditioned stimulus or CS), with an innately noxious stimulus (designated the unconditioned stimulus or US). If this association is successfully learned and remembered, the previously neutral CS will by itself elicit a fear response (designated the conditioned response or CR) appropriate to the original noxious US. Normally, the CS-US association is readily acquired and the memory and subsequent CR can persist for years without further reinforcement. Disruption of learning and memory by pharmacological agents can occur at the acquisition, consolidation, reconsolidation and extinction phases of classical fear conditioning.

Associative learning is a critical survival tool and as such the underlying mechanism for classical fear conditioning is highly conserved across species. Animals as diverse as fruit flies and humans can be conditioned using very similar procedures (95). In experimental classical fear conditioning, the CS can be almost any discrete non-threatening cue such as a tone, light, or scent; the US is noxious or mildly painful. Generally, in vertebrates, the US can be as simple as a puff of air into the face or a brief electric shock. Auditory

conditioning, where the CS-US is a tone-shock pairing is most frequently used in rodents (mice and rats) and is described in this chapter.

The neural circuitry underlying auditory fear conditioning is the most studied and best understood of the fear conditioning paradigms. In auditory fear conditioning, a commonly used CS is a neutral non-noxious sound, using a single frequency tone (78; 95; 108). Auditory signals reach the lateral amygdala via two routes: a direct thalamoamygdala route and indirectly via the auditory cortex (78). Evidence indicates that synaptic plasticity at these synapses underlies the formation of an auditory conditioned fear memory (19; 72; 78). Behavioral expression of conditioned fear memory requires an intact central nucleus of the amygdala (37; 38; 123; 175). Learned associations of the CS with the training context require synaptic input from the hippocampus [1,3]. Thus intact hippocampal function is essential for contextual components of fear conditioning (60; 104; 128). Synaptic input from the prefrontal cortex is required both for the extinction of conditioned fear memories (112; 115; 170) and for the behavioral expression of conditioned fear (40; 115; 160). Behavioral changes in the components of fear conditioning can therefore indicative of function in the areas of the brain from which they originate.

Following classical fear conditioning, physiological and behavioral indicators of fear are expressed upon presentation of the CS. Physiologic changes include activation of the hypothalamic pituitary adrenal (HPA) axis, and the autonomic

nervous system (ANS) (31; 139; 165). A key behavioral response to the CS is freezing behavior. Freezing is an innate defensive behavior leading to the cessation of all movement except as required for breathing (32; 39). This is a well validated indicator of fear in rodents (24; 25). Freezing behavior is an ideal behavior to measure because it is quantifiable, easy to obtain through direct observation and measurement can be automated. Below we describe, common rodent subjects, apparatus and methods to perform fear conditioning and for analyzing freezing behavior.

2. Materials

Rodents as subjects

All strains of rats and mice can be used for classical fear conditioning. An important consideration for fear conditioning and behavioral testing in general is the age of the rodent at the time of experimentation. Most experiments and pharmacological manipulations are carried out in adult animals. Fear conditioning can be performed on animals of any age and learning can occur. However, in very young and adolescent animals the learning and memory phenotype is quite different from adult animals (32; 162). For general phenotyping of learning and memory deficits or enhancement, adult animals (generally between 8 and 16 weeks of age) are used

Rodent subjects should be housed under uniform conditions with respect to lighting, temperature, and potential stressors, such as cage changes, housing room traffic, or other disturbances (132). Animals ordered into the facility from a laboratory animal supplier should be allowed to habituate to their new housing conditions for a week or more. Unless circumstances dictate single housing (such as post-surgical animals or highly aggressive animals) rodents should not be singly housed as this may have adverse effects on behavioral results (157; 171). Rats are generally housed two or three per cage while mice may be housed up to 5 per cage. With aggressive strains of mice, such as C57/B6 housing male animals fewer than 5 to a cage may be optimal. Enrichment items such as pressed cotton nesting material or rodent chew toys may also be provided and should meet institutional guidelines. It should be pointed out however, that enrichment facilitates coping and resilience to stress (10; 114) and may be problematic in studies attempting to evaluate consequences of stress on behavior and brain function or in studies along similar lines.

All experiments conducted on animals must be reviewed and approved by an Institutional Animal Care and Use Committee. The numbers of animals required per experimental group should be determined by power analysis. Generally 7-10 rats per condition and 10-15 mice per condition provide sufficient statistical power. The example experiments described here were conducted on adult male

(8-10 weeks old) Sprague-Dawley rats and adult (8-12 weeks old) C57/B6 x DBA/J2 hybrid male and female mice.

Equipment needed for laboratory based Pavlovian Fear Conditioning

The primary and essential components for fear conditioning are a conditioning chamber with an electrical conduction rod floor, an electrical shock generator, a tone generator, cameras and recording equipment, as well as a sound attenuation box to isolate sounds, especially an auditory CS, within the conditioning chamber. Additional components include alternate flooring and wall panels to change the appearance of the testing environment (Figure 1); and software for automated scoring of freezing behavior if desired. Fear conditioning chambers are available from many companies who manufacture behavioral testing apparatus. In the following experiments, fear conditioning apparatus for rats were manufactured by Coulbourn Instruments (Whitehall, PA, USA). Videos of the testing were digitally recorded for manual scoring. Mouse experiments were conducted in Coulbourn Habitest chambers and scored using Coulbourn's FreezeFrame® software. The mouse chambers are 7in wide by 7in deep by 12in high with ¼ in conducting rods spaced ½ in apart. Rat chamber dimensions are 12in wide by 10in deep by 12in high with ¼ in conducting rods spaced ¾ in apart. Specific chamber dimensions can vary, however, they must be appropriate for

the animal (e.g. grid floor spacing is wider for rats) and allow for the ability to move and explore.

Equipment needed for Measurement of Freezing Behavior

Pavlovian fear conditioning allows the investigator to obtain quantitative measures of conditioned fear memory. A well characterized response to conditioned fear in rodents is "freezing". Freezing is an innate defensive behavior leading to the cessation of all movement, excluding respiration. Freezing behavior is an ideal behavior to measure because it is quantifiable, easy to obtain and measurement can be automated. Measures of freezing are obtained for each subject by visual observation and/or automated scoring of freezing. Scoring of freezing by a human observer should be consistent and reproducible. Variability between human scorers can occur, thus ideally only one person should score each data set. If this is not possible, or the data set is large, internal controls for consistency should be used.

For high throughput analysis, freezing behavior can be scored using automated scoring programs. Commercially available automatic freeze scoring software, include FreezeFrame® available through Coulbourn Instruments, (Whitehall, PA USA) and AnyMaze® software available through Stoelting (Wood Dale, IL USA). Mousemove (described in (87)) is a free program for scoring freezing behavior available for download from the Malinow group at Cold Spring Harbor

Laboratories (http://malinowlab.cshl.edu/downloads/.) Computerized systems combine the sensitivity and objectivity desired for unbiased data. FreezeFrame®, and most automated scoring software for freezing, is not a tracking system, but rather, a motion detection system that is capable of detecting movements as small as 1mm. This is accomplished by calculating motion in a given digital image detected over a period of time and given a score based on pixels. Then, two successive images are subtracted and if the pixel score equals zero (0), no motion occurred. In contrast, if a non-zero score is calculated between two successive images, it is due to motion. The animal is monitored several times each second and so the ability to reliably detect fine movements of the head and limbs is possible and consistent between animals. Additionally, FreezeFrame® is capable of scoring freezing in low-light levels and will filter shadows or artifact from electronic equipment.

Measures of freezing calculated by any automated system should be compared to values determined by the human investigator in pilot trials. Whatever method of scoring is chosen, it is critical that the investigator ensures consistency across groups of subjects. It is highly advisable to video-record each aspect of the experiment so that the investigator has the ability to verify freezing scores and analyze multiple aspects of behavior.

In the experiments described below, using rats as subjects, freezing behavior was manually scored from recorded video, by an observer blind to the training

conditions. Data from experiments using mouse subjects was scored using Freezeframe automated scoring software available from Coulbourn Instruments (Whitehall, PA, USA). Freezeframe allows the user to define freezing. This is done by setting the threshold of maximum movement (number of pixels changing) and the minimum duration for which the number of pixels changing must remain below threshold, a temporal parameter termed "bout". The freezing threshold was determined by a trained researcher from digital playback of recorded training sessions in the Freezeframe program and the minimum bout duration was set at 0.25 seconds.

3.Methods

Fear Conditioning Protocol

A 'classical' fear conditioning protocol involves 4 components: **habituation, training, context memory test and cue (CS) memory test** (See Figure 1 and data in Figures 2 and 3). An additional component of extinction of the conditioned response can be included as well. Extinction is a new learning process in which upon repeated presentation of the CS in the absence of the US the subject learns that the CS no longer predicts the US. Subsequent to this new learning, the CS no longer generates the conditioned response. For each phase of fear conditioning, pertinent data should be collected. Baseline measurements of movement and anxiety can be obtained prior to training, either over a number of

days during habituation or on the training day prior to the first CS-US presentation. Some automated scoring programs include a tracking component, which will measure actual distance traveled during the test or segments of the test, but determining baseline freezing is sufficient to indicate potential confounding differences in pre-training activity. This is particularly important under circumstances where experimental manipulations may be sedating, cause hyperactivity, impair locomotor function and balance (which may make movement on the grid floor more difficult) and for obtaining a complete phenotype of genetically modified animals.

Habituation

Habituating animals to the conditioning chambers as well as to human handling, transport and other aspects of the experimental procedures is important for minimizing unconditioned freezing. In the example experiments, rats were handled daily for a total of 5 days in an effort to reduce the stress of human contact. To reduce baseline freezing (154), rats were habituated to the conditioning chamber each day for 20 minutes for 3 consecutive days (Figure 2A). Rats readily adjust to being handled whereas mice do not. Therefore, mice were not handled prior to the habituation-training day (Figure 3A). With repeated handling, mice may begin to show signs of chronic stress (99). Habituation of mice consisted of 3 minutes in the chamber on the training day before the onset of the first tone. On each day of the fear conditioning protocol, both rats and

mice were transported from the LAM facility to a holding room and allowed a minimum of 20-30 minutes to recover from the stress of transportation.

Training

In delay conditioning, the conditioned stimulus is contingent in time with the unconditioned stimulus. In the example experiments, a 30 second 5 kHz, 75 dB pure tone conditioned stimulus co-terminates with a brief foot-shock delivered through the conducting rod floor. Schematics of the timeline for each experiment are provided in figures 2 and 3. In both experiments, subjects were allowed 3 minutes in the chamber prior to the first CS-US presentation (Figure 2A and Figure 3A). In experiments with 3 or more CS-US presentations, the interval between CS-US presentations was variable to prevent the subject from anticipating CS onset. In the experiments described in figure 2, the interval varied between 90 and 180s with a mean of 120s. In the 10 x 0.5 mA condition described in figure 3, the inter-trial interval varied between 15 and 50 seconds with a mean of 30s. Within an experiment, each subject received the same pattern of CS-US presentations. In the rat experiments (Figures 2 and 4) the US was delivered for 0.5s at 0.6 mA current. In the mouse experiments (Figure 3) the US was delivered for 2s at either 0.5 mA or 1.0 mA current as described.

Learning can be compared between experimental and control conditions for rate of acquisition and maximal freezing (Figure 3B). When using mild shock as the unconditioned stimulus, differences in pain threshold, which will affect the rate of acquisition, extent of freezing, and subsequent memory test should be accounted

for. Again this is particularly important when experimental manipulations may have analgesic (or hyperalgesic) effects and in transgenic animals. The strength of learning and the resultant memory can be easily manipulated by varying the training intensity either through the number of CS-US presentations or the intensity of the US (Figure 2B). It is generally a good idea to run a pilot experiment to determine a protocol which giving an appropriate level of freezing in the memory tests for the control condition. When there is no a priori hypothesis as to whether the experimental treatment will facilitate or impair memory formation, freezing in the control condition should fall into a median range of 50-60%. If the treatment is expected to facilitate memory formation then a less rigorous training protocol will allow more sensitive detection of increases in freezing behavior. In the reverse condition when treatment impairs fear memory, a more rigorous training protocol may be desirable.

Context Memory

Testing context memory involves simply returning the subject to the training environment and measuring fear behavior. In this test, rats were returned to the training chamber for 15 minutes 1 day after fear conditioning training (Figure 2). Freezing can be scored over the duration of the test or in smaller bins of time across the test. In this example, freezing was scored for the first 20 seconds of each minute (Figure 2B). In longer tests, particularly those that are being manually scored, scoring shorter bins of time may be more time efficient. In our

experience, scoring 20 seconds of each minute is representative of freezing across the entire test. Furthermore, by breaking up longer context tests into bins of time, the reduction of fear (context extinction) over time, when no CS or US is presented, can be determined (Figure 4A).

Cue Memory

To test learning and memory to the CS component of conditioned fear (tone in the example experiments), testing must be conducted in an environment free (to the greatest practical extent) of contextual reminders of the training (27). The goal is to have a pure measure of the strength of the tone-shock association. Ideally, testing could occur in completely novel chambers, however this is not usually practical and the training chambers are used for the cue-test. In this case, as many features of the testing environment are changed as possible. Changes include the chamber dimensions or geometry, flooring, lighting, cleaning solution and other olfactory cues, adding patterns to the chamber walls and if possible changing the lighting conditions and visual cues in the testing room itself (Figure 1).

In the rat experiments, testing for cued fear memory was conducted in a second chamber novel to the rat subjects. Lighting conditions, box geometry, flooring surface (smooth surface with sawdust bedding as opposed to the conducting rod floor), wall color and olfactory cues differed from the training chamber. The

animals were allowed three minutes to explore the new chamber. After three minutes, the same 30s 5kHz tone used in the fear conditioning training was presented to the animals. Over the course of the test (20 minutes), the CS was presented 10 times with a variable inter-trial interval. The inter-trial interval was between 90 seconds and 180 seconds with a 120 second mean but was a different pattern from that used in fear conditioning training. Freezing behavior was scored for the initial 3 minute exploration period and for each CS presentation.

In the mouse experiments, the testing chambers were altered to provide the new environment for the cue test (Figure 1). The conducting rod floors were replaced with a mesh grid floor and white paper used to line the catch pan underneath. Graphic black and white patterns were applied to the walls of the conditioning chambers. Strips of acetate were slotted into the interior of the chamber to further change the visual appearance. Yellow acetate filters were placed over the house lights to change the lighting effect. A 1% acetic acid solution was used to mask the previous cleaning solution and provide new olfactory cues. Lights in the testing room were dimmed from the training day to change the appearance of the testing room. In these experiments as well, subjects were allowed a three minute exploration period in the chamber prior to the first CS presentation. The CS was presented to the animals twice with a 30 second interval. Freezing was measured during the initial 3 minute period and for each presentation of the CS.

Freezing to the first CS presentation is the purest indicator of the conditioned response to the auditory stimulus, without the influence of preceding stimuli. Frequently however, multiple tones are presented during the course of the test and total cue-associated freezing is reported. Data presented in figure 2B is the mean freezing across all tone presentations for each training condition. Additionally, in tests where the CS is presented many times, within-trial extinction to the un-reinforced conditioned stimulus can be evaluated (Figure 4B.)

Several minutes of exploration should be allowed prior to the first presentation of the CS, and freezing should be measured during that time (Figure 3C). Comparisons of pre-training freezing with post-training freezing in a novel environment may indicate fear generalization or stress-induced neophobia. However, in situations where the training environment is masked to produce the alternate context for cue testing, context bleed-through due to inadequate masking of the training environment should be considered.

4. Notes

The experiments described above are tests of long-term memory (LTM), that is, the animals are tested after the period of time required for new protein synthesis and stabilization of learning and short term memory (STM) into enduring memory (82). Pavlovian fear conditioning of the rodent has revealed a discrete time-

course for such changes believed to be 4-6 hours for the consolidation of training into long-term memory (151; 153). The mechanisms of STM and LTM are distinct. Short-term memory involves activation of cAMP second messenger systems and short term synaptic remodeling independent of new protein synthesis (70). Long term memory formation is dependent on new protein synthesis in the amygdala (11; 151; 152). It should be noted that experimental treatments frequently have differential effects on short term and long term memory (70; 151). Pavlovian fear conditioning can be used to test either STM or LTM based upon whether conditioning training and subsequent context and cue testing occur within the 4-6 hour consolidation window or beyond it. Caution should be used when testing both short and long term memory in the same cohort of animals. Behavior is influenced by previous experience and as a result, tests for STM may influence behaviors on any subsequent behavioral tests such as those for LTM.

Within a given experiment, make every effort to test subjects at the same time of the day. While fear conditioning is an exceptionally robust behavioral paradigm, circadian rhythms in hormonal and molecular systems influencing learning and memory may introduce subtle phenotypic changes and variability (36; 54; 62). The majority of researchers train and test animals housed on a standard 12:12 light cycle (lights on during the day) with good results.

Researchers conducting behavioral experiments must make an effort to control as many variables as possible and no amount of attention to detail is too much. Subtle factors such as odors (e.g. perfumes, fear pheromones, the smell of other animals) or extraneous noise (mechanical equipment, construction, nearby vocalization) in the housing, holding or testing rooms potentially impact the results. To that end, it is important to thoroughly clean the chambers (particularly the grid flooring) between subjects with an appropriate cleaning solution (e.g. 70% ethanol, 1% acetic acid) and testing rooms should be clean and away from high-traffic locations. Having low volume (around 60dB) background noise in the training/testing chamber may help obscure unavoidable noise outside and is frequently either a ventilation fan in the sound attenuation box or a scrambled frequency white noise produced from the sound generator. The holding room should be in close proximity to the testing room, but situated where the animals cannot hear the auditory cue prior to training or to testing. Pre-exposure to the CS will adversely affect the learning of the CS-US association through a process called latent inhibition (100).

Ideally, stressors such as cage changes should not coincide with a testing day. Likewise, if animals were without water during a prolonged period of time (e.g. overnight due to water bottle leakage) and/or were subjected to wet bedding, the resulting stress to the animal may adversely affect behavioral results. Maintaining a detailed lab notebook with anticipated and unanticipated aspects or

occurrences of an experiment will aid the investigator in data analysis and alert the research group to factors that potentially played a role in their results.

Figure 1

A

B

Figure 1. A: Context "A" used for training and context test. B: Context "B" used for cue testing.

Figure 2

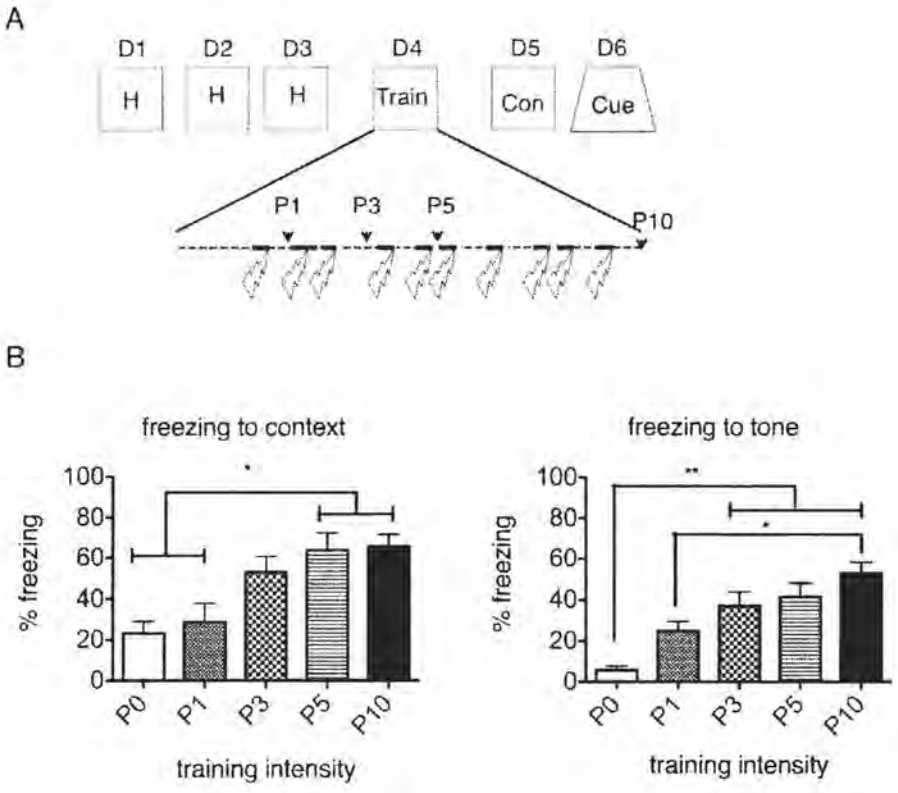

A
D1
D2
D3
D4
D5
D6
H
H
H
Train
Con
Cue
P1
P3
P5
P10
B
freezing to context
% freezing
100
80
60
40
20
0
P0
P1
P3
P5
P10
training intensity
freezing to tone
% freezing
100
80
60
40
20
0
P0
P1
P3
P5
P10
training intensity

Figure 3

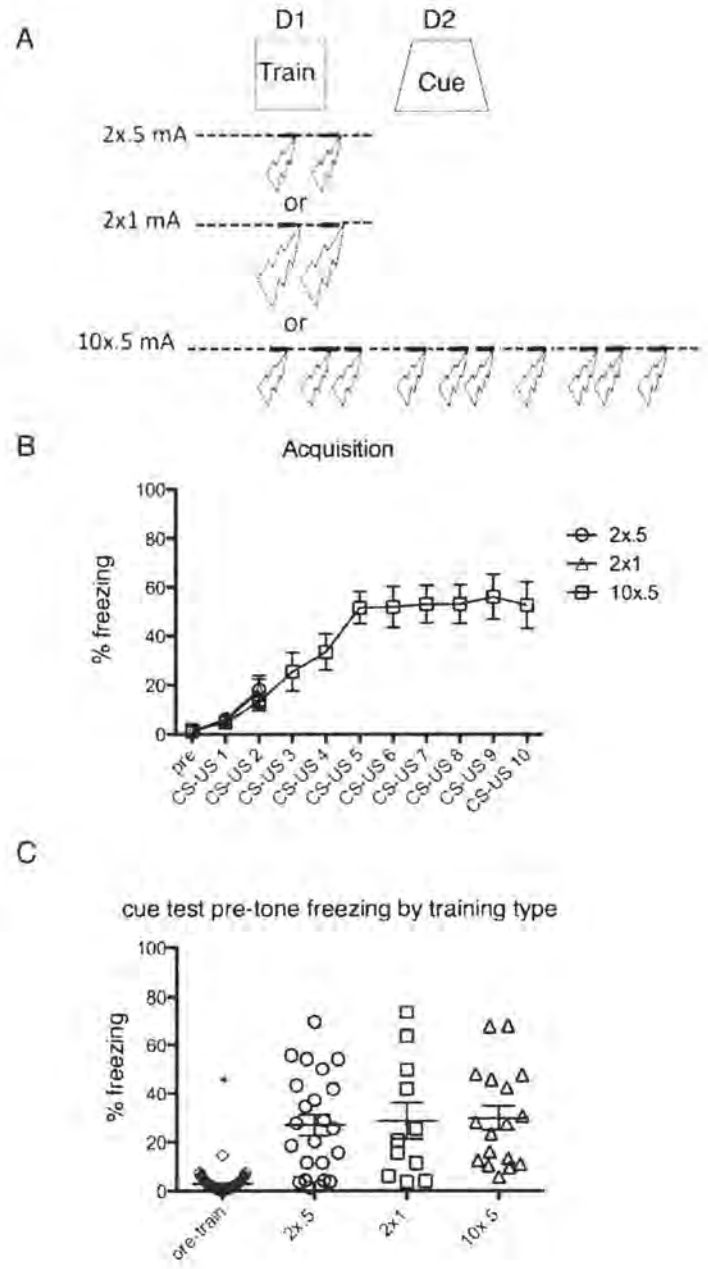
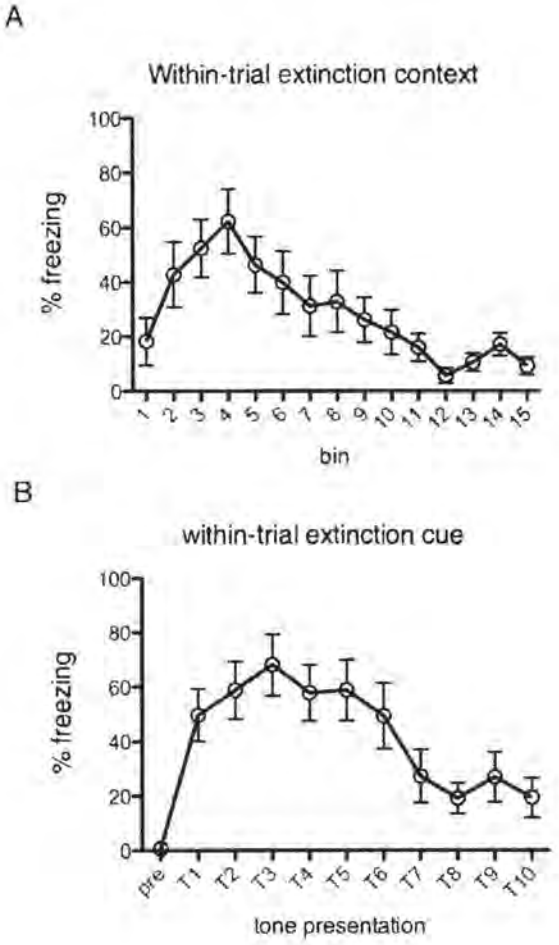

A
D1
Train
D2
Cue
2x.5 mA
or
2x1 mA
or
10x.5 mA
B
Acquisition
% freezing
100
80
60
40
20
0
2x.5
2x1
10x.5
pre
CS-US 1
CS-US 2
CS-US 3
CS-US 4
CS-US 5
CS-US 6
CS-US 7
CS-US 8
CS-US 9
CS-US 10
C
cue test pre-tone freezing by training type
% freezing
100
80
60
40
20
0
pre-train
2x.5
2x1
10x.5

Figure 4


A
Within-trial extinction context
% freezing
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
bin
B
within-trial extinction cue
% freezing
100
80
60
40
20
0
pre T1 T2 T3 T4 T5 T6 T7 T8 T9 T10
tone presentation

REFERENCES

1. Aggleton JP. 1992. *The Amygdala : neurobiological aspects of emotion, memory, and mental dysfunction*. New York: Wiley-Liss. xii, 615 p. pp.

2. Aggleton JP. 2000. *The amygdala : a functional analysis*. Oxford, OX ; New York: Oxford University Press. xiv, 690 p. pp.

3. Alberts B, Johnson A, Lewis J, Raff M. *Molecular Biology of the Cell*

pp 15. Garland Science Taylor & Francis Group

4. Amaral DG, Price, D.L., Pitkanen, A., and Carmichael, S.T. 1992. *The Amygdala: Neurobiological Aspects of Emotion, Memory, and Mental Dysfunction*. pp 1-66.

5. Anagnostaras SG, Maren S, Fanselow MS. 1999. Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 19:1106-14

6. Association AP. 1994. *Diagnostic and Statistical Manual of Mental Disorders*.

7. Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD. 1998. The MAPK cascade is required for mammalian associative learning. *Nature neuroscience* 1:602-9

8. Bailey CH, Bartsch D, Kandel ER. 1996. Toward a molecular definition of long-term memory storage. *Proceedings of the National Academy of Sciences of the United States of America* 93:13445-52

9. Balogh SA, Radcliffe RA, Logue SF, Wehner JM. 2002. Contextual and cued fear conditioning in C57BL/6J and DBA/2J mice: context discrimination and the effects of retention interval. *Behavioral neuroscience* 116:947-57

10. Belknap JK, Richards SP, O'Toole LA, Helms ML, Phillips TJ. 1997. Short-term selective breeding as a tool for QTL mapping: ethanol preference drinking in mice. *Behavior genetics* 27:55-66

11. Benedek DM. 2011. Posttraumatic stress disorder from Vietnam to today: the evolution of understanding during Eugene Brody's tenure at the journal of nervous and mental disease. *The Journal of nervous and mental disease* 199:544-52

12. Bergstrom HC, McDonald CG, Dey S, Tang H, Selwyn RG, Johnson LR. 2012. The structure of Pavlovian fear conditioning in the amygdala. *Brain structure & function*

13. Bergstrom HC, McDonald CG, Johnson LR. 2011. Pavlovian fear conditioning activates a common pattern of neurons in the lateral amygdala of individual brains. *PloS one* 6:e15698

14. Besnard A, Laroche S, Caboche J. 2013. Erratum to: Comparative dynamics of MAPK/ERK signalling components and immediate early genes in the hippocampus and amygdala following contextual fear conditioning and retrieval. *Brain structure & function*

15. Black AH, Young GA. 1972. Electrical activity of the hippocampus and cortex in dogs operantly trained to move and to hold still. *Journal of comparative and physiological psychology* 79:128-41

16. Blair HT, Schafe GE, Bauer EP, Rodrigues SM, LeDoux JE. 2001. Synaptic plasticity in the lateral amygdala: a cellular hypothesis of fear conditioning. *Learn Mem* 8:229-42

17. Blanchard DC, Blanchard RJ. 1972. Innate and conditioned reactions to threat in rats with amygdaloid lesions. *Journal of comparative and physiological psychology* 81:281-90

18. Blanchard DC, Blanchard RJ. 1988. Ethoexperimental approaches to the biology of emotion. *Annual review of psychology* 39:43-68

19. Blanchard DC, Hynd AL, Minke KA, Minemoto T, Blanchard RJ. 2001. Human defensive behaviors to threat scenarios show parallels to fear- and anxiety-related defense patterns of non-human mammals. *Neuroscience and biobehavioral reviews* 25:761-70

20. Blanchard RJ, Blanchard DC. 1969. Passive and active reactions to fear-eliciting stimuli. *Journal of comparative and physiological psychology* 68:129-35

21. Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31-9

22. Bonanno GA, Galea S, Bucciarelli A, Vlahov D. 2007. What predicts psychological resilience after disaster? The role of demographics, resources, and life stress. *Journal of consulting and clinical psychology* 75:671-82

23. Bordi F, LeDoux J. 1992. Sensory tuning beyond the sensory system: an initial analysis of auditory response properties of neurons in the lateral amygdaloid

nucleus and overlying areas of the striatum. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 12:2493-503

24. Brewin CR. 2008. What is it that a neurobiological model of PTSD must explain? *Progress in brain research* 167:217-28

25. Bryant RA. 2003. Early predictors of posttraumatic stress disorder. *Biological psychiatry* 53:789-95

26. Bush DE, Sotres-Bayon F, LeDoux JE. 2007. Individual differences in fear: isolating fear reactivity and fear recovery phenotypes. *Journal of traumatic stress* 20:413-22

27. Carter M, Shieh, J. 2010. *Guide to Research Techniques in Neuroscience*. Elsevier

28. Choi JS, Brown TH. 2003. Central amygdala lesions block ultrasonic vocalization and freezing as conditional but not unconditional responses. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23:8713-21

29. Coyner J, McGuire, J., Parker, C., Ursano, R., Palmer, A., Johnson, LR. 2013. Mice selectively bred for High and Low fear behavior show differences in the number of pMAPK (p44/42 ERK) expressing neurons in lateral amygdala following Pavlovian fear conditioning. *Neurobiology of learning and memory*

30. Cukor J, Spitalnick J, Difede J, Rizzo A, Rothbaum BO. 2009. Emerging treatments for PTSD. *Clinical psychology review* 29:715-26

31. Davis M. 1992. The role of the amygdala in fear and anxiety. *Annual review of neuroscience* 15:353-75

32. Davis M. 1997. Neurobiology of fear responses: the role of the amygdala. *The Journal of neuropsychiatry and clinical neurosciences* 9:382-402

33. Davis M. 2000. *The role of the amygdala in conditioned and unconditioned fear and anxiety, in The Amygdala: A functional Analysis*. pp 213-287.

34. Davis M, Whalen PJ. 2001. The amygdala: vigilance and emotion. *Molecular psychiatry* 6:13-34

35. Davis S, Laroche S. 2006. Mitogen-activated protein kinase/extracellular regulated kinase signalling and memory stabilization: a review. *Genes, brain, and behavior* 5 Suppl 2:61-72

36. Debiec J, Bush DE, LeDoux JE. 2011. Noradrenergic enhancement of reconsolidation in the amygdala impairs extinction of conditioned fear in rats--a

possible mechanism for the persistence of traumatic memories in PTSD. *Depression and anxiety* 28:186-93

37. Delgado MR, Olsson A, Phelps EA. 2006. Extending animal models of fear conditioning to humans. *Biological psychology* 73:39-48

38. Di Benedetto B, Kallnik M, Weisenhorn DM, Falls WA, Wurst W, Holter SM. 2009. Activation of ERK/MAPK in the lateral amygdala of the mouse is required for acquisition of a fear-potentiated startle response. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology* 34:356-66

39. Duvarci S, Nader K, LeDoux JE. 2005. Activation of extracellular signal-regulated kinase- mitogen-activated protein kinase cascade in the amygdala is required for memory reconsolidation of auditory fear conditioning. *The European journal of neuroscience* 21:283-9

40. Ehlers A, Clark DM. 2000. A cognitive model of posttraumatic stress disorder. *Behaviour research and therapy* 38:319-45

41. Elzinga BM, Bremner JD. 2002. Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)? *Journal of affective disorders* 70:1-17

42. Epstein RS, Fullerton CS, Ursano RJ. 1998. Posttraumatic stress disorder following an air disaster: a prospective study. *The American journal of psychiatry* 155:934-8

43. Fanselow MS. 1980. Conditioned and unconditional components of post-shock freezing. *The Pavlovian journal of biological science* 15:177-82

44. Fanselow MS, Kim JJ. 1994. Acquisition of contextual Pavlovian fear conditioning is blocked by application of an NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid to the basolateral amygdala. *Behavioral neuroscience* 108:210-2

45. Fendt M, Fanselow MS. 1999. The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience and biobehavioral reviews* 23:743-60

46. Franklin K, Paxinos, G. 2008. *The Mouse Brain in Stereotaxic Coordinates*. Elsevier

47. Heim C, Nemeroff CB. 2009. Neurobiology of posttraumatic stress disorder. *CNS spectrums* 14:13-24

48. Hoge CW, Castro CA, Messer SC, McGurk D, Cotting DI, Koffman RL. 2004. Combat duty in Iraq and Afghanistan, mental health problems, and barriers to care. *The New England journal of medicine* 351:13-22

49. Hoge CW, Terhakopian A, Castro CA, Messer SC, Engel CC. 2007. Association of posttraumatic stress disorder with somatic symptoms, health care visits, and absenteeism among Iraq war veterans. *The American journal of psychiatry* 164:150-3

50. Holmes A, Murphy DL, Crawley JN. 2003. Abnormal behavioral phenotypes of serotonin transporter knockout mice: parallels with human anxiety and depression. *Biological psychiatry* 54:953-9

51. Holmes A, Quirk GJ. 2010. Pharmacological facilitation of fear extinction and the search for adjunct treatments for anxiety disorders--the case of yohimbine. *Trends in pharmacological sciences* 31:2-7

52. Humeau Y, Herry C, Kemp N, Shaban H, Fourcaudot E, et al. 2005. Dendritic spine heterogeneity determines afferent-specific Hebbian plasticity in the amygdala. *Neuron* 45:119-31

53. JC E. 1987. Mechanisms of Learning in Complex Neural Systems. In *Handbook of Physiology*, ed. F Plum, 5:137-67: American Physiological Society. Number of 137-67 pp.

54. Johnson LR, Hou M, Ponce-Alvarez A, Gribelyuk LM, Alphs HH, et al. 2008. A recurrent network in the lateral amygdala: a mechanism for coincidence detection. *Frontiers in neural circuits* 2:3

55. Johnson LR, Hou M, Prager EM, Ledoux JE. 2011. Regulation of the Fear Network by Mediators of Stress: Norepinephrine Alters the Balance between Cortical and Subcortical Afferent Excitation of the Lateral Amygdala. *Frontiers in behavioral neuroscience* 5:23

56. Johnson LR, LeDoux JE. 2004. The anatomy of fear: microcircuits of the lateral amygdala. *In Fear and Anxiety: The Benefits of Translational Research, APPA Press, Washington DC*:227-50

57. Johnson LR, Ledoux JE, Doyere V. 2009. Hebbian reverberations in emotional memory micro circuits. *Frontiers in neuroscience* 3:198-205

58. Johnson LR, McGuire J, Lazarus R, Palmer AA. 2012. Pavlovian fear memory circuits and phenotype models of PTSD. *Neuropharmacology* 62:638-46

59. Josselyn SA, Shi C, Carlezon WA, Jr., Neve RL, Nestler EJ, Davis M. 2001. Long-term memory is facilitated by cAMP response element-binding protein

overexpression in the amygdala. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21:2404-12

60. Jovanovic T, Ressler KJ. 2010. How the neurocircuitry and genetics of fear inhibition may inform our understanding of PTSD. *The American journal of psychiatry* 167:648-62

61. Kandel ER. 2000. *Principles of Neural Science.* McGraw-Hill Companies, Inc.

62. Kandel ER. 2001. The molecular biology of memory storage: a dialogue between genes and synapses. *Science* 294:1030-8

63. Kessler RC. 2000. Posttraumatic stress disorder: the burden to the individual and to society. *The Journal of clinical psychiatry* 61 Suppl 5:4-12; discussion 3-4

64. Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB. 1995. Posttraumatic stress disorder in the National Comorbidity Survey. *Archives of general psychiatry* 52:1048-60

65. Kim JJ, Fanselow MS. 1992. Modality-specific retrograde amnesia of fear. *Science* 256:675-7

66. Kim JJ, Jung MW. 2006. Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. *Neuroscience and biobehavioral reviews* 30:188-202

67. LaBar KS, Gatenby JC, Gore JC, LeDoux JE, Phelps EA. 1998. Human amygdala activation during conditioned fear acquisition and extinction: a mixed-trial fMRI study. *Neuron* 20:937-45

68. LaBar KS, LeDoux JE, Spencer DD, Phelps EA. 1995. Impaired fear conditioning following unilateral temporal lobectomy in humans. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15:6846-55

69. Lamprecht R, LeDoux J. 2004. Structural plasticity and memory. *Nature reviews. Neuroscience* 5:45-54

70. LeDoux J. 2000. The amygdala and emotion: a view through fear. In *The Amygdala: A Functional Analysis*, ed. JP Aggleton:289-310. Number of 289-310 pp.

71. LeDoux JE. 1992. Brain mechanisms of emotion and emotional learning. *Current opinion in neurobiology* 2:191-7

72. LeDoux JE. 1994. Emotion, memory and the brain. *Scientific American* 270:50-7

73. LeDoux JE. 1996. *The emotional brain : the mysterious underpinnings of emotional life.* New York: Simon & Schuster. 384 p. pp.

74. LeDoux JE. 2000. Emotion circuits in the brain. *Annual review of neuroscience* 23:155-84

75. LeDoux JE. 2002. *Synaptic self : how our brains become who we are.* New York: Viking. x, 406 p. pp.

76. LeDoux JE, Cicchetti P, Xagoraris A, Romanski LM. 1990. The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 10:1062-9

77. Lissek S, Powers AS, McClure EB, Phelps EA, Woldehawariat G, et al. 2005. Classical fear conditioning in the anxiety disorders: a meta-analysis. *Behaviour research and therapy* 43:1391-424

78. Magruder KM, Yeager DE. 2008. Patient factors relating to detection of posttraumatic stress disorder in Department of Veterans Affairs primary care settings. *Journal of rehabilitation research and development* 45:371-81

79. Maren S. 1996. Synaptic transmission and plasticity in the amygdala. An emerging physiology of fear conditioning circuits. *Molecular neurobiology* 13:1-22

80. Maren S. 2001. Neurobiology of Pavlovian fear conditioning. *Annual review of neuroscience* 24:897-931

81. Maren S, Aharonov G, Fanselow MS. 1997. Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. *Behavioural brain research* 88:261-74

82. Maren S, Fanselow MS. 1995. Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 15:7548-64

83. Maren S, Quirk GJ. 2004. Neuronal signalling of fear memory. *Nature reviews. Neuroscience* 5:844-52

84. Mazzucchelli C, Brambilla R. 2000. Ras-related and MAPK signalling in neuronal plasticity and memory formation. *Cellular and molecular life sciences : CMLS* 57:604-11

85. McGaugh JL, Introini-Collison IB, Nagahara AH, Cahill L, Brioni JD, Castellano C. 1990. Involvement of the amygdaloid complex in neuromodulatory influences on memory storage. *Neuroscience and biobehavioral reviews* 14:425-31

86. Milad MR, Rauch SL, Pitman RK, Quirk GJ. 2006. Fear extinction in rats: implications for human brain imaging and anxiety disorders. *Biological psychology* 73:61-71

87. Morgan MA, Romanski LM, LeDoux JE. 1993. Extinction of emotional learning: contribution of medial prefrontal cortex. *Neuroscience letters* 163:109-13

88. Muigg P, Hetzenauer A, Hauer G, Hauschild M, Gaburro S, et al. 2008. Impaired extinction of learned fear in rats selectively bred for high anxiety--evidence of altered neuronal processing in prefrontal-amygdala pathways. *The European journal of neuroscience* 28:2299-309

89. Norrholm SD, Jovanovic T, Olin IW, Sands LA, Karapanou I, et al. 2011. Fear extinction in traumatized civilians with posttraumatic stress disorder: relation to symptom severity. *Biological psychiatry* 69:556-63

90. Olff M, Langeland W, Gersons BP. 2005. The psychobiology of PTSD: coping with trauma. *Psychoneuroendocrinology* 30:974-82

91. Orr SP, Lasko NB, Macklin ML, Pineles SL, Chang Y, Pitman RK. 2012. Predicting post-trauma stress symptoms from pre-trauma psychophysiologic reactivity, personality traits and measures of psychopathology. *Biology of mood & anxiety disorders* 2:8

92. Palmer AA, Phillips TJ. 2002. Effect of forward and reverse selection for ethanol-induced locomotor response on other measures of ethanol sensitivity. *Alcoholism, clinical and experimental research* 26:1322-9

93. Pape HC, Pare D. 2010. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiological reviews* 90:419-63

94. Parker CC, Sokoloff G, Cheng R, Palmer AA. 2012. Genome-wide association for fear conditioning in an advanced intercross mouse line. *Behavior genetics* 42:437-48

95. Paylor R, Tracy R, Wehner J, Rudy JW. 1994. DBA/2 and C57BL/6 mice differ in contextual fear but not auditory fear conditioning. *Behavioral neuroscience* 108:810-7

96. Peri T, Ben-Shakhar G, Orr SP, Shalev AY. 2000. Psychophysiologic assessment of aversive conditioning in posttraumatic stress disorder. *Biological psychiatry* 47:512-9

97. Phelps EA, Delgado MR, Nearing KI, LeDoux JE. 2004. Extinction learning in humans: role of the amygdala and vmPFC. *Neuron* 43:897-905

98. Phillips RG, LeDoux JE. 1992. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. *Behavioral neuroscience* 106:274-85

99. Pitkanen A. 2000. *Connectivity of the rat amygdaloid complex. In: The Amygdala a functional analysis*. pp 31-115.

100. Pitkanen A, Savander V, LeDoux JE. 1997. Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. *Trends in neurosciences* 20:517-23

101. Ponder CA, Kliethermes CL, Drew MR, Muller J, Das K, et al. 2007. Selection for contextual fear conditioning affects anxiety-like behaviors and gene expression. *Genes, brain, and behavior* 6:736-49

102. Prigerson HG, Maciejewski PK, Rosenheck RA. 2001. Combat trauma: trauma with highest risk of delayed onset and unresolved posttraumatic stress disorder symptoms, unemployment, and abuse among men. *The Journal of nervous and mental disease* 189:99-108

103. Quirk GJ, Repa C, LeDoux JE. 1995. Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat. *Neuron* 15:1029-39

104. Rauch SL, Whalen PJ, Shin LM, McInerney SC, Macklin ML, et al. 2000. Exaggerated amygdala response to masked facial stimuli in posttraumatic stress disorder: a functional MRI study. *Biological psychiatry* 47:769-76

105. Repa JC, Muller J, Apergis J, Desrochers TM, Zhou Y, LeDoux JE. 2001. Two different lateral amygdala cell populations contribute to the initiation and storage of memory. *Nature neuroscience* 4:724-31

106. Rescorla RA. 1968. Probability of shock in the presence and absence of CS in fear conditioning. *Journal of comparative and physiological psychology* 66:1-5

107. Richter-Levin G. 2004. The amygdala, the hippocampus, and emotional modulation of memory. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* 10:31-9

108. Rodrigues SM, Schafe GE, LeDoux JE. 2004. Molecular mechanisms underlying emotional learning and memory in the lateral amygdala. *Neuron* 44:75-91

109. Rogan MT, LeDoux JE. 1996. Emotion: systems, cells, synaptic plasticity. *Cell* 85:469-75

110. Rolls ET. 1990. Theoretical and neurophysiological analysis of the functions of the primate hippocampus in memory. *Cold Spring Harbor symposia on quantitative biology* 55:995-1006

111. Romanski LM, Clugnet MC, Bordi F, LeDoux JE. 1993. Somatosensory and auditory convergence in the lateral nucleus of the amygdala. *Behavioral neuroscience* 107:444-50

112. Romanski LM, LeDoux JE. 1992. Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 12:4501-9

113. Romanski LM, LeDoux JE. 1993. Information cascade from primary auditory cortex to the amygdala: corticocortical and corticoamygdaloid projections of temporal cortex in the rat. *Cereb Cortex* 3:515-32

114. Rosen JB, Donley MP. 2006. Animal studies of amygdala function in fear and uncertainty: relevance to human research. *Biological psychology* 73:49-60

115. Rudy JW, O'Reilly RC. 2001. Conjunctive representations, the hippocampus, and contextual fear conditioning. *Cognitive, affective & behavioral neuroscience* 1:66-82

116. Rumpel S, LeDoux J, Zador A, Malinow R. 2005. Postsynaptic receptor trafficking underlying a form of associative learning. *Science* 308:83-8

117. Sanders MJ, Wiltgen BJ, Fanselow MS. 2003. The place of the hippocampus in fear conditioning. *European journal of pharmacology* 463:217-23

118. Schafe GE, Atkins CM, Swank MW, Bauer EP, Sweatt JD, LeDoux JE. 2000. Activation of ERK/MAP kinase in the amygdala is required for memory consolidation of pavlovian fear conditioning. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20:8177-87

119. Schafe GE, LeDoux JE. 2000. Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20:RC96

120. Schafe GE, Nadel NV, Sullivan GM, Harris A, LeDoux JE. 1999. Memory consolidation for contextual and auditory fear conditioning is dependent on protein synthesis, PKA, and MAP kinase. *Learn Mem* 6:97-110

121. Schafe GE, Nader K, Blair HT, LeDoux JE. 2001. Memory consolidation of Pavlovian fear conditioning: a cellular and molecular perspective. *Trends in neurosciences* 24:540-6

122. Seger R, Krebs EG. 1995. The MAPK signaling cascade. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 9:726-35

123. Selcher JC, Atkins CM, Trzaskos JM, Paylor R, Sweatt JD. 1999. A necessity for MAP kinase activation in mammalian spatial learning. *Learn Mem* 6:478-90

124. Shiromani PJ, LeDoux JE, Keane TM. 2009. *Post-traumatic stress disorder : basic science and clinical practice*. New York: Humana Press. xiii, 409 p. pp.

125. Sotres-Bayon F, Cain CK, LeDoux JE. 2006. Brain mechanisms of fear extinction: historical perspectives on the contribution of prefrontal cortex. *Biological psychiatry* 60:329-36

126. Squire LR. 1987. The Nervous System: Higher Functions of the Brain. In *Handbook of Physiology*, ed. F Plum, 5:295-371: American Physiological Society. Number of 295-371 pp.

127. Stein DJ. 2009. The psychobiology of resilience. *CNS spectrums* 14:41-7

128. Stiedl O, Radulovic J, Lohmann R, Birkenfeld K, Palve M, et al. 1999. Strain and substrain differences in context- and tone-dependent fear conditioning of inbred mice. *Behavioural brain research* 104:1-12

129. Sweatt JD. 2004. Mitogen-activated protein kinases in synaptic plasticity and memory. *Current opinion in neurobiology* 14:311-7

130. Treves A, Rolls ET. 1994. Computational analysis of the role of the hippocampus in memory. *Hippocampus* 4:374-91

131. Turner BH, Mishkin M, Knapp M. 1980. Organization of the amygdalopetal projections from modality-specific cortical association areas in the monkey. *The Journal of comparative neurology* 191:515-43

132. Ursano RJ, Zhang L, Li H, Johnson L, Carlton J, et al. 2009. PTSD and traumatic stress from gene to community and bench to bedside. *Brain research* 1293:2-12

133. Watson JB, Rayner, R. 1920. Conditioned emotional reactions. *Journal of Experimental Psychology* 3:1-14

134. Weiskrantz L. 1956. Behavioral changes associated with ablation of the amygdaloid complex in monkeys. *Journal of comparative and physiological psychology* 49:381-91

135. Whalen PJ, Johnstone T, Somerville LH, Nitschke JB, Polis S, et al. 2008. A functional magnetic resonance imaging predictor of treatment response to venlafaxine in generalized anxiety disorder. *Biological psychiatry* 63:858-63

136. Whalen PJP, E. 2009. *The Human Amygdala*. The Guilford Press

137. Yehuda R. 2004. Risk and resilience in posttraumatic stress disorder. *The Journal of clinical psychiatry* 65 Suppl 1:29-36

138. Yehuda R, LeDoux J. 2007. Response variation following trauma: a translational neuroscience approach to understanding PTSD. *Neuron* 56:19-32